MPHIL

A Population-Based Approach to the Study of Virulence Determinants in Methicillin-Resistant Staphylococcus Aureus

Ouadi, Khadija

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A Population-Based Approach to the Study of Virulence Determinants in Methicillin-Resistant *Staphylococcus aureus*

Khadija Ouadi

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ACKNOWLEDGEMENTS

I'd like to start this report, if I may, with my Acknowledgements section. As the people I would like to address here have done so much for me, it seems only fair that they're addressed first. Before I jump into the extensive list of the people that made this MPhil report possible, I'd like to start by opening up a little. I hid a lot of things during my time at Bath. I'm not proud to admit it, but hiding those things in part contributed in a progressive decline in my mental health. Opening up to someone a bit sooner might have made a difference to my standard of work, however it would have first required me to have admitted to myself that I was struggling in the first place – I guess I missed a pretty clear window of opportunity there..! I applaud the recent work the Department has done in order to address postgraduate mental health (and I feel that it would be remiss of me to not mention my lab-mate Emily Stevens specifically for being so proactive in her involvement in it during her time at Bath PGBio – and with the amount of work you generated during your PhD, quite frankly, I don't know how you did it all!); I really hope these efforts continue.

In the weeks preceding the end to my funded lab work, I was diagnosed with a form of PTSD: a diagnosis that was very freeing in a lot of ways. It gave me the permission to accept that the work submitted here would not be of PhD standard. It's somewhat of a bitter pill to swallow; the projects that make up this collective dataset each had substantial promise in the right hands. I just wish I had fewer other things to juggle at the same time – I imagine I would have enjoyed the process a lot more and have gained a lot more from it. I owe a lot of people an explanation for some of my (if you'll forgive the euphemism) interesting decisions made during my time at Bath. With your support, and enough time to recover, I now have the hindsight needed to see that I wasn't exactly the most reliable student, and for that, I apologise. As somebody that used to shudder at the very thought of handing in work that I wasn't completely happy with, my sudden shift in attitude towards my work was confusing to say the least. I'm sure my undergraduate self would be pretty shocked to learn that her later attempts to submit a PhD thesis would not only be a Master's, but one that required an extension of all things!

While the work carried out was by no means flawless, there is still merit in it. From an educational stand point, there are a lot of aspects to the experimental method presented here that I would have approached completely differently now. The retrospective analysis of

negative or incomplete data is a useful exercise in itself – if nothing else, I hope others gain something from it. From a personal perspective, however, it presents my efforts during my time at the university and gives some degree of purpose to my own personal struggles that coincided with my studies. To the people I address here: thank you for being the motivating factor for me submitting this document.

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TABLE OF CONTENTS

ABBREVIATIONS

HAI	Healthcare-associated infection
MLST	Multilocus sequence typing
WGS	Whole genome sequencing
GWAS	Genome-wide association study
MRSA	Methicillin-resistant *Staphylococcus aureus*
SCC*mec*	Staphylococcal cassette chromosome *mec*
MGE	Mobile genetic element
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
PVL	Panto-Valentine leukocidin
PSM	Phenol soluable modulins
TSST-1	Toxic shock syndrome toxin 1
CA-MRSA	Community-associated MRSA
HA-MRSA	Hospital-acquired MRSA
SNP	Single nucleotide polymorphism
INDEL	Insertion/deletion
CGH	Comparative genome hybridization
EPS	Extracellular polymeric substance
PIA	Polysaccharide intracellular adhesin
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
PBS	Phosphate buffered saline
OD	Optical density
HCW	Healthcare workers
CC	Clonal complex
ST	Sequence type
PTS	Phosphotransferase system
BCAA	Branch chained amino acids
IL	Interleukin
TNF	Tumor necrosis factor

eDNA	Environmental DNA
IRAS	Integrated Research Application System
SSI	Site-Specific Investigation
TCDA	10,12-Tricosadiynoic acid
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DPPTE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
CF	5(6)-carboxyfluorescein
LB	Lysogeny broth
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
sRNA	Regulatory RNA
MDA	Multiple displacement amplification
RT-PCR	Reverse transcription PCR
NMR	Nuclear magnetic resonance

TABLE OF FIGURES

ABSTRACT

This study aims to provide insights into the application of next generation sequencing for the study of hospital-acquired staphylococcal infections. *Staphylococcus aureus* is a gram-positive, commensal species of bacteria that are a common constituent of the human microbiome. However, many of the strains of this opportunistic pathogen, the causative agent of a wide range of infections, have now acquired resistance to many frequently used antibiotics. At its worst, the threat of antimicrobial resistance could result in increased rates of post-surgical mortality for even routine surgical procedures and, generally, make clinical facilities potential epicentres for infection outbreaks.

With that in mind, this body of work will focus largely on methicillin resistant *S. aureus* (MRSA), given its relevance in clinical environments currently. A metagenomic approach was used in an attempt to monitor the transmission of microbial pathogens upon the relocation of a paediatric burns unit from one site to another, however an unfortunate technical oversight prevented with project from being completed.

In the study of an epidemic strain ST228, an attempt was also made at establishing any causal relationships between bacterial phenotypes and their ability to disseminate to the scale of an outbreak, however no inferences could be gleaned from the study of biofilm formation, toxin production and phage susceptibility in select isolates from the ST228 cohort, warranting further study.

Finally, a GWAS was carried out on a cohort of ST239 isolates for biofilm formation and protease activity. The protease GWAS identified potential associations with certain loci in the TW20 reference genome, however either the lack of sensitivity of the assay used or inherent differences between the proteolytic potential of ST239 and the USA300_FPR3757 strain that made up the available transposon mutant library meant that the dataset was ultimately inconclusive. Five genes (*mecA*, *sgaT*, *ccrB, aap* and *hyp*) were identified as potentially being associated to biofilm formation in ST239. Verification of these associations through complementation was unsuccessful. Ultimately, despite some technical issues, the analysis of whole genomic sequence data combined with the screening of a large collection of ST239 isolates identified a handful of potentially novel effectors of biofilm formation that warrant additional characterization in the future

INTRODUCTION

STAPHYLOCOCCUS AUREUS AND NOSOCOMIAL INFECTIONS

The Burden of Nosocomial Infection

Nosocomial infections, also known as hospital-acquired infections (HAIs), are a leading cause of patient death globally, with an estimated 4% of acute-care patients contracting at least one during admission in the US (Hassan Ahmed Khan, Ahmad and Mehboob, 2015). The developing world is hit hardest however, with the average number of nosocomial infections increasing to 40% (Hassan Ahmed Khan, Ahmad and Mehboob, 2015). Globally, this can in part be attributed to aging populations and, on average, larger hospitals, with central catheter insertion and long admission times being key risk factors for later infections (Lax and Gilbert, 2015). In addition to causing significant morbidity and mortality, nosocomial infections are estimated to cost US hospitals $4.5 billion dollars annually (Reed and Kemmerly, 2009). The infections causes by these pathogens vary widely, with some of the most frequent infection types including central line-associated bloodstream infections, catheter associated urinary tract infections and surgical site infections (Khan, Baig and Mehboob, 2017). A particular group of common nosocomial pathogens, the "ESKAPE pathogens" (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species specifically), have the additional challenge of often being resistant to a number of commonly used antimicrobials (Boucher *et al.*, 2009). This report will focus on one of the leading causative agents of HAIs, *S. aureus*. Annually, in the US alone, *S. aureus* is responsible for over 20,000 deaths (Thomer *et al.*, 2016) – costing more lives annually in the US than HIV/AIDS (Peschel and Otto, 2013) .

Staphylococcus aureus

Bacteria within the genus *Staphylococcus* are gram-positive, catalase-positive cocci that aggregate into characteristic grape-like clusters. This genus can be subdivided into 42 species, of which 10 can be subdivided into subspecies. Individual isolates within the species *S. aureus* can be further characterized and distinguished through multilocus sequence typing (MLST), where isolates are distinguished from one another through sequence variation of internal fragments of seven housekeeping genes. These sequence variations can then be assigned as

distinct alleles from which further clonal groups, known as clonal complexes (CCs) and sequence types (STs), can be formed (Enright *et al.*, 2000).

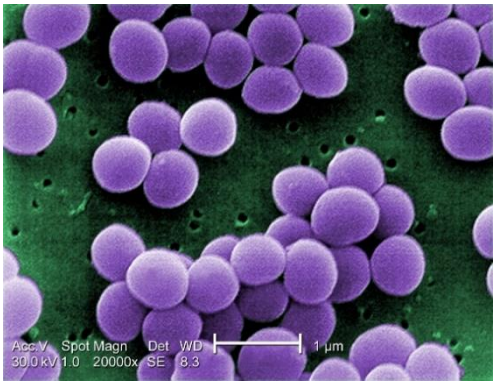

Figure 1- An SEM image of the characteristic grape-like clusters of S. aureus (Janice Haney Carr, CDC)

Bacteria in the genus *Staphylococcus* are largely opportunistic pathogens, with many acting as commensal bacteria on the skin of a host until an opportunity for sub-cutaneous invasion presents itself (such as via indwelling medical devices or the colonisation of a wound surface) (Ghebremedhin *et al.*, 2008). This genus contains one of the most frequently isolated species of pathogens for clinical samples, *Staphylococcus aureus* (Kobayashi, Malachowa and DeLeo, 2015).

S. aureus (Figure 1) is one of the most common bacterial pathogens worldwide, responsible for a myriad of infections (Otto, 2013). While around 30% of the population are intermittently colonised by the bacteria, and 20% of the population permanently colonised (Otto, 2010), this usually benign constituent of the human microbiome is also the causative agent of numerous skin and soft tissue infections, as well as bacteremia, infective endocarditis and pulmonary infections. As such, this pathogen is of global importance as a leading cause of morbidity and mortality, particularly in clinical setting.

Methicillin-resistant *S. aureus*

MRSA has been a significant burden to healthcare providers for many years, with the first incidence of nosocomial MRSA infections occurring in 1985 (Farr *et al.*, 2001). Following the sudden appearance of this pathogen in the media and public consciousness, various infection control management strategies were implemented in the UK in the early twentieth century to reduce the transmission frequency of this high profile 'superbug'. In 2013, a high-profile statement from Professor Dame Sally Davies (Chief Medical Officer for England) described microbial antibiotic resistance to be 'as big a risk as terrorism', stating that routine operations could become life-threatening in as little as 20 years' time. However, despite media attention having somewhat moved away from MRSA and other nosocomial infections, the emergence of epidemic strains of these common nosocomial pathogens is a reoccurring challenge to modern clinical microbiology. Active surveillance screening has been used in various countries

as a means of preventing exogenous transmission of the pathogen, as increasing hand hygiene and contact isolation alone has previously been described as inadequate in controlling the spread of nosocomial pathogens. However, given that we've seen a 24.5% increase in MRSA cases of bacteremia since these reports were made mandatory, more still needs to be done (Thelwall *et al.*, 2017).

MRSA, as with any isolate of *S. aureus*, often causes relatively minor skin and soft tissue infections. Yet, when a breach exists within a potential hosts immune defenses, this opportunistic pathogen is given the opportunity to become a causative agent of a host of life-threatening infections, including bacteraemia, pneumonia and endocarditis (Bamberger, 2007). In the case of MRSA, resistance for widely used beta-lactam antibiotics is gained through the acquisition of the mobile genetic element (MGE) SCC*mec* and the well-characterised *mecA* gene carried within it (Chongtrakool *et al.*, 2006). Worryingly, vancomycin, a common choice of treatment for MRSA infections, may be also be rendered ineffective by the emergence of vancomycin resistance in *S. aureus*, which was first reported in 2002 (Chang *et al.*, 2003). Despite the relatively recent gains made through the discovery of antibiotics, our species arms race against our natural pathogens is not as close-run as we might like to believe.

Within this report, two STs of MRSA will be investigated further, ST228, otherwise known as the Southern Germany clone (Mick *et al.*, 2010), and ST239, one of the most predominate clones of MRSA in nosocomial infections (Abimanyu, Murugesan and Krishnan, 2012).

PATHOGENESIS IN *S. AUREUS*

S. aureus and MRSA infections vary so significantly in part due to the number of genes involved in the intricate regulation of *S. aureus* pathogenesis, allowing for its rapid replication and dissemination to a range of sites. Potential disease manifestations including infective endocarditis, organ tissue abscesses and sepsis (Peschel and Otto, 2013) . Understanding the mode through which *S. aureus* exerts its pathogenicity is essential for improving our treatment of the many infections caused by it.

Human skin is the first line of defense of the immune system: a layer of keratinocytes serving as a physical barrier to prevent the entry of invading bacterial pathogens into deeper layers of tissue. In instances where there has been a traumatic breach of this barrier, a complex cellular

response occurs to mobilise immune cells to the site of infection (Kobayashi, Malachowa and DeLeo, 2015). However, once that initial breach is made, whether the resulting infection is benign or life threatening depends, in part, on which virulence factors the strain possesses. Virulence factors, molecules that increase the likelihood of a pathogen causing disease, are often encoded on MGEs, which are able to then be transferred from one strain to another through horizontal gene transfer (Otto, 2014).

The phenotypic plasticity of *S. aureus* makes this species particularly interesting to study, since the propensity of this pathogen for transferring virulence genes has given rise to the abundance of virulence factors these bacteria are able to employ. The expression of the vast set of genes that are responsible for exerting *S. aureus* virulence is highly regulated through the action of a variety of regulatory genes (Figure 2), the combined actions of which determine the multitude of pathogenic phenotypes seen *in vitro*.

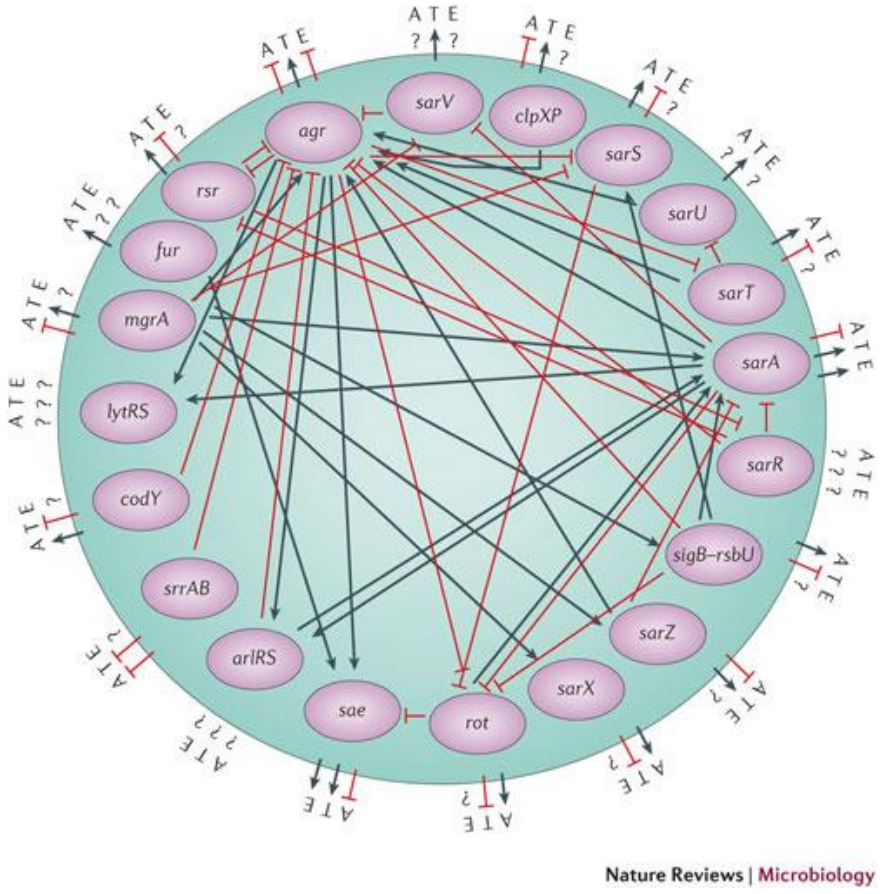

Figure 2 A schematic representation of the regulatory genes in S. aureus that interact to induce either upregulation or downregulation of adhesin (A), evasin (E) or toxicity (T) genes (Recker et al., 2017)

Together the differential expression of the arsenal of accessory genes that govern *S aureus* pathogenicity, government by the various proteins and RNA molecules that make up the *S. aureus* virulence regulatory network, produce strain-specific virulence profiles, with some strains being more invasive or toxic than others (Otto, 2014).

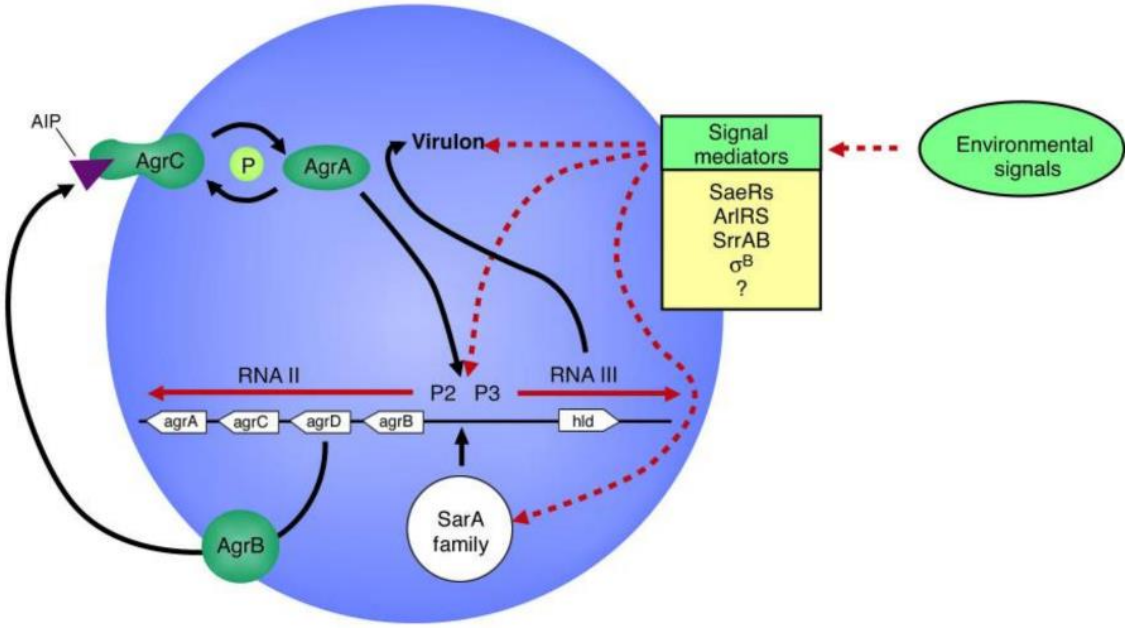

Figure 3 The accessory gene regulator (agr) system in Staphylococcus (Jeremy M. Yarwood and Schlievert, 2003)

One of the best characterized gene systems through which *S. aureus* pathogenicity is moderated is through the global regulator *agr*. This operon is a quorum-sensing system enabling cell-to-cell communication and is made up of divergent transcription units that comprises five separate *agr* genes that result in the production of RNAIII, an effector molecule for a number of downstream gene targets responsible for the production of colonisation and virulence factors (Novick, 2003) (Figure 3). The *agr* operon coordinates the differential expression of cell-surface adhesion proteins, exogenous toxins and evasin genes in response to cell population density – for example, when a bacterial population reaches around late exponential/early growth phase, adhesins are downregulated whilst toxins such as staphylokinase, hemolysins, protein A and toxin shock syndrome toxin-1 (TSST-1) are upregulated (Pragman and Schlievert, 2004) . The *agr* system is also responsible for epithelial cell invasion and apoptosis (Jeremy M. Yarwood and Schlievert, 2003). When *agr* is downregulated, *S. aureus* persists as a coloniser of the human epidermis and other surfaces

through the action of wall teichoic acid mediated adherence, and then the binding of microbial surface components recognizing matrix molecules (MSCRAMMs) to the mammalian extracellular matrix, allowing the pathogen to adhere to its host and, ultimately, colonise host tissues (Christopher *et al.*, 1999).

Another well-characterised regulator of *S. aureus* virulence factors is the staphylococcal accessory regulator (SarA), a DNA-binding protein expressed from three promotors that binds to both the P2 and P3 promoters of the *agr* system. However, *sarA* plays a role in regulating other virulence factors of its own, including downregulating protease activity, activating α-hemolysin and staphylococcal enterotoxin B expression and binding to fibronectin-binding protein and staphylococcal enterotoxin C promotors (Pragman and Schlievert, 2004).

These are just two of the most well-known regulators within the *S. aureus* genomes – a drastic simplification of the complexity of the interactions of the regulator genes involved in determining the expression levels of evasins, toxins and adhesins. They act in concert to determine a plethora of important, clinically relevant phenotypes.

S. aureus virulence determinants

A wealth of pathogenic determinants exists which, collectively, mediate *S. aureus* tissue colonization and tissue damage. Adhesins, for instance, facilitate the pathogens adherence to host cells and largely include a family of molecules called MSCRAMMs. MSCRAMMs include important molecules for cell surface attachment, including clumping factors and fibrinogen-binding proteins (Spellberg and Daum, 2012). Meanwhile leukocidins such as Panto-Valentine leukocidin (PVL), an example of an evasin, interfere with the host's immune system (Eap) (Spellberg and Daum, 2012). Additionally, a variety of toxins, molecular agents that directly harm the host, as well as biofilm production and resistance to antibiotics all work in concert to exert *S. aureus* pathogenicity. An overview of the various virulence factors in various infections can be seen in Figure 4.

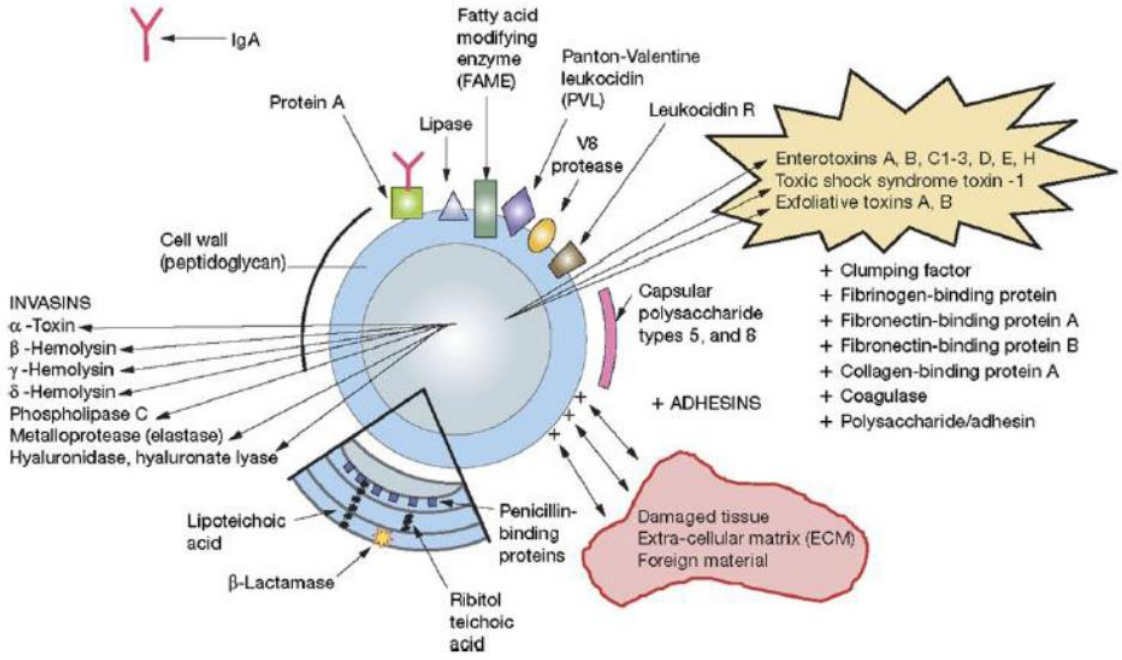

Figure 4 The various virulence factors employed by S. aureus during infection (Spellberg and Daum, 2012)

Staphylococcal toxins

The broad range of staphylococcal toxins, which facilitate damage to host tissue during an infection, can be grouped into three distinct categories:

1. Membrane-damaging or cytolytic toxins: these produce breaks in host cell membranes that lead to the efflux of vital metabolites and ultimately lyse the cell. These include α-toxin, which produced pores within the membranes of erythrocytes and several leukocytes (Otto, 2014).
2. Toxins that inhibit the action of host cell receptors: these include the staphylococcal enterotoxins, a class of superantigens responsible for disrupting intestinal function, and TSST, a superantigen which stimulates the release of numerous cytokines, including interleukin (IL) -1 and -2 and tumour necrosis factor (TNF) α (Otto, 2014).
3. Secreted enzymes: through degrading host molecules, this class of toxin can interfere with host cell metabolism and signalling cascades. These include a class of enzymes known as proteases. *S. aureus* has the ability to synthesis and secrete 10 major proteases; aureolysin (aur), V8 (SspA) serine protease, staphopains A and B (ScpA and SspB respectively) and six separate serine-like SspA homologues (SplABC-DEF) (Kolar

et al., 2013). These proteases interact in an interdependent post-translational activation cascade to exert a variety of vital roles, including the modulation of surface adhesive molecules of the bacteria, resulting in a shift from an adhesive phenotype to an invasive one, disseminating the pathogen further (Shaw *et al.*, 2004). A cascade of serine proteases specifically plays a role in coagulation through the enzymatic conversion of prothrombin to thrombin and the cleavage of fibrinogen into soluble fibrin (Friedrich *et al.*, 2003). In addition, the proteolytic activity of these enzymes have been attributed to serpin inactivation (a protein important in processes including coagulation and inflammatory immune responses), the degradation of various extracellular matrix components and prothrombin activation (another important protein in host coagulation)(Shaw *et al.*, 2004), suggesting that the cumulative action of these proteolytic enzymes play active roles in both invasive phenotypes, dissemination and evasion of innate host immunity.

Biofilm formation

Staphylococci, along with many other genera of bacteria, can establish dynamic social agglomerations known as biofilms, in which multiple bacterial cells are encased within an extracellular polymeric substance (EPS). Biofilm formation is in many ways a more advantageous state than the planktonic, single-cell state of singular bacteria, particularly for colonisers of mammalian skin such as *S. aureus*, which not only protects bacteria from drastic environmental change or being washed off a surface, but also provides protection from innate host defense mechanisms and antibiotics (Thurlow *et al.*, 2011). Although there is a certain degree of uncertainty and controversy surrounding what constitutes a biofilm, it is becoming increasingly evident that nosocomial bacterial infection commonly involves biofilms, particularly in cases involving the insertion of indwelling medical devices and wound infections (Archer *et al.*, 2011). Within these bacterial aggregates, biofilm formation is modulated elegantly through the sophisticated quorum sensing *agr* operon (the aforementioned master-controller of the transcription of virulence factors) (J. M. Yarwood *et al.*, 2004). *S. aureus* biofilms are embedded within glycocalyx (a network of polysaccharide fibers that extend out from the cell wall), formed after the initial adhesion of *S. aureus* to a surface in the first step of biofilm development; attachment. This is facilitated through non-covalent interactions between the surface-attached adhesins such as MSCRAMMs and host tissue/abiotic surfaces (Otto, 2008).

Following attachment, proliferation and maturation of the biofilm occurs. This involves the production of an extracellular matrix consisting of polysaccharide intracellular adhesins (PIAs), teichioc acids, environmental DNA (eDNA) and proteins with intricate channels formed to mediate the transfer to nutrients through the layers of the biofilm. Once the biofilm has developed sufficiently, clusters of the biofilm detach in order to be disseminated to distal locations within the host (Le *et al.*, 2014) (Figure *3*).

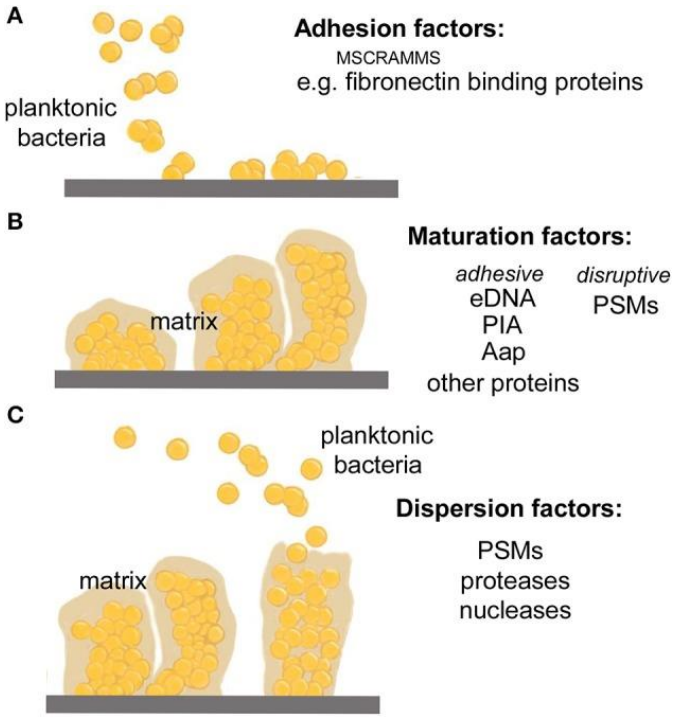

Figure 3 The stages of biofilm development in *S. aureus* (Le *et al.*, 2014)

Bacteriophage-resistance

Bacteriophages (shortened to phages hereafter), an entity even more abundant than bacteria, are bacterial viruses that need to infect a bacterial host in order to replicate, making them an interesting alternative therapeutic agent for infections caused by multidrug resistant bacteria (Sulakvelidze, Alavidze and Morris, 2001). The microflora of the human body is known to include an abundance of various bacteriophages, which are putatively regarded as important for maintaining the body's regular microbial ecology (Clokie *et al.*, 2011). Given their abundance, several pathogens, *S. aureus* included, have evolved resistance against lytic phage. However, a trade-off between this resistance and the production of other virulence determinants can occur (León and Bastías, 2015), thus making phage resistance an interesting phenotype to compare alongside toxin production and biofilm formation.

INFECTION CONTROL IN THE POST-GENOMIC ERA

The advent of high throughput DNA sequencing technology has resulted in various paradigm shifts in classical microbiology: one of the many facets of the life sciences expanded during the genomic era. Conventional bacterial classifications based on bacterial colony morphology increasingly gave way to species identification through 16S ribosomal RNA sequencing. Since then, species and clonal variant identification has steadily improved, with whole-genome mapping steady increasing in resolution, owing to the dramatic improvement of both speed and affordability that modern sequencing methods have to offer. In the last few decades, the number of bacterial genomes fully sequenced has grown almost exponentially (Land *et al.*, 2015), and this trend is set to continue, with technologies such as hand-held sequencing platforms promising instantaneous sequencing already commercially available.

In the case of infection and immunity, whole-genome sequencing (WGS) of pathogens within clinical samples is often used to provide insights into the genomic epidemiology of bacteria commonly responsible for nosocomial infections – a practice made easier by the fact that these samples are routinely collected already within clinical environments. Various clinical infection control initiatives exist to curb the likelihood of infecting new patients according to what is already known about how pathogens spread in clinical environments. For example, the hands of healthcare workers (HCWs) are routinely cited as one of the most common vehicles by which microorganisms are transmitted from one patient to another (Saloojee and Steenhoff, 2001). Additionally, other putative modes of transmission for common nosocomial pathogens include HCW clothing/personal items (such as stethoscopes and personal phones) and fomites in clinical environments including computer keyboards and medical devices (Lax and Gilbert, 2015). While poor infection control practices do increase the risk of nosocomial infection, good hygiene practices sadly are not enough to prevent microbial transmission from staff to patients. It is estimated that between 4-6% of healthcare workers in any one hospital harbour nosocomial pathogens on their skin, with the risk of carriage by healthcare workers elevated by comorbidities (such as recent urinary tract infections and cystic fibrosis), recent antibiotic use, previous work abroad and high work load (Albrich and Harbarth, 2008). That said, however, infections are by and large also transfected through either direct or in direct contact between patients, visitors and environmental sources (Boucher *et al.*, 2009). Ultimately, our current knowledge of how to curb the spread of pathogens in healthcare environments needs to improve to provide efficient and reliable infection control strategies.

This report presents a body of work exploring several approaches that utilise our recent advances in genomics: metagenomics, a "phenotype-first" approach to studying epidemics and genome-wide association studies (GWAS) as a tool for identifying novel candidate genes associated to clinically relevant phenotypes.

Metagenomics

Increasing our understanding of the intricate molecular processes occurring within populations of bacteria that are representative of clinical environments is of paramount importance. Effective infection control requires knowledge of the relationship between environmental contamination and incidence of patient infection, the elucidation of potential candidate genes involved in sudden incidences of hospital epidemics and new insights into unexplained changes in an infected patients morbidity.

Our lack of a concrete understanding of the population dynamics of clinical pathogens can, in part, be attributed to studies carried out previously that focused on known pathogens and a reliance on microbial cultures. A strong argument can be made for the utility of metagenomic sequencing in the monitoring of microbial populations within clinical settings. Assessing the whole microbiome of an environmental sample offers a viable alternative to the 'needle in a haystack' approach to microbial epidemiology (via selecting for specific nosocomial pathogens of interest), which often fails to give a true picture of microbial diversity (Lax and Gilbert, 2015). Such approaches have already been carried out in the development of a sequence-based diagnosis tool for wound and post-surgical infection (Chan and Chan, 2013). Microbial metagenomic analysis of clinical environments are only beginning to emerge within the literature, with the first reported use of real-time metagenomic next-generation sequencing to influence infection control occurring in 2017 (Greninger *et al.*, 2017).

For clinical or environmental samples with relatively complex microbiomes, the faithful detection and identification of the species diversity within that sample can be difficult to ascertain using conventional sequencing methods, such as Sanger sequencing of specific pathogens individually (Nakamura *et al.*, 2008). With newly developed next-generation DNA sequencers able to sequence over 100 megabases of DNA per run (Nakamura *et al.*, 2008), shotgun metagenomics has emerged as a successful alternative to conventional sequencing. This method allows for the untargeted sequencing of all the genomes present in a sample, providing insights into the taxonomic composition of the microbial community whilst also

recovering whole genomic sequences. Metagenomic approaches to the study of a samples microbiome may therefore provide a more accurate impression of the full species diversity within a sample, including those that are frequently difficult to culture in laboratories (Quince *et al.*, 2017). Shotgun metagenomics may be used to overcome a number of challenges to the field of clinical microbiology and, with time, could provide more accurate diagnoses and therapeutic strategies, informed by a comprehensive understanding of the genome of the causative agent, can be tailored to maximise the efficacy of any subsequent treatment.

The first aim of this project was to survey any changes to the microbial burden of a burns unit caused by its relocation. The relocation of the Bristol Children's burns unit in 2014 presented an exciting opportunity to employ metagenomics to studying changes to a wards microbial ecology when a clinical environment moves geographically. Chapter 1 details the collection of environmental samples from a children's burns unit in Bristol (UK), a hospital used for casualties during World War II, to a larger unit within a newer hospital that was deep-cleaned before the move. From this, it was intended that various common nosocomial pathogens from a single environmental sample could be pooled and sequenced in tandem to illustrate the potential changes to transmission dynamics seen within a children's burns unit and the effect that a ward relocation has on species diversity.

Deriving molecular explanations for outbreaks from strain phenotyping

In studies centered around community associated MRSA (CA-MRSA), the striking ability of this pathogen to evade host immunity so effectively and spread so easily has been attributed to either the action of PSMs or PVLs (Otto, 2012). However, in HA-MRSA (hospital-acquired MRSA) limited information currently exists regarding staphylococcal phenotypes that play a role in establishing an outbreak, which can result in instances where, despite adhering to common infections controls, outbreak can occur suddenly and dramatically. Lausanne University Hospital, for instance, saw an unexpected spike in infections caused by MRSA-ST228 in 2008, after previous outbreaks of ST228 in 2001 and 2003 seemed to be mitigated by the infection control efforts of the hospital (Vogel *et al.*, 2012). The second aim of this project, described in Chapter 2, was to explore why the 2008 outbreak occurred through a study of phenotypic differences in strains collected throughout all three outbreak periods. Given the unusual pattern of ST228 outbreaks seen at this hospital and the fact that previous genomic analysis of the isolates failed to explain the reasons for this outbreak, a few

phenotypes of interest were studied within ST228 isolates taken various years between 2000 and 2009, with a view towards potentially correlating a given phenotype with high incidences of ST228 infections – a "phenotype-first" look at epidemics that may suggest that factors outside the genome (for instance, post-translational modifications) could be responsible for the outbreak periods observed. The subset of ST239 strains chosen from the Lausanne outbreak strain collection were tested for biofilm formation, susceptibility to lytic phage and δ -toxin and PSM production.

Genome-wide association studies

The advent of novel high-throughput genotyping makes genome-wide association studies (GWAS) an attractive approach to studying populations of pathogens within clinical environments. GWASs test large numbers of genetic variants (including whole genes, single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) within a population for statistically significant associations to a certain phenotype (Read and Massey, 2014). This bottom-up approach to identifying the molecular basis of phenotypes begins with a specific phenotype of interest and associates any differences in phenotype within a population with particular loci in the pan-genome (Falush and Bowden, 2006). This technique, which was first employed as a way of extrapolating Mendelian principles to complex inherited disorders in humans (Visscher *et al.*, 2012), has shown a comparatively muted level of interest within microbiology. This is in spite of the fact that, owing to the smaller genome of bacteria, the consensus base-calling accuracy is usually higher in bacterial GWAS, given that the average coverage of Illumina shotgun projects is rarely less than 20 (Read and Massey, 2014). The concept of correlating phenotype to the bacterial genome is not a new one; comparative genome hybridization (CGH) microarrays were previously used for this exact purpose. However, GWASs have the additional benefits of facilitating the discovery of new genes, whilst insights into genomic diversity is limited by the reference sequences used in the design of CGH array probes (Dutilh *et al.*, 2013).

Previous data from our lab has validated the utility of GWAS in developing our understanding of the molecular processes that government infectious disease. This technique has been used to develop a predictive model for predicting an isolates toxicity from its genetic signature and phenotypic data (M. Laabei *et al.*, 2014b) for a Turkish collection of MRSA-ST239 isolates. Through assaying large number of clonally related isolates, inferences have also been made

regarding the high degree of phenotypic variability within this clone (Maisem Laabei and Massey, 2016). The final aim of this project was to study biofilm formation and protease production using GWAS, with the hope of uncovering novel genetic loci associated with these phenotypes. Chapter 3 explores the data gained through screening the Turkish ST239 strain collection for phenotypic variation, determining potential candidate genes through statistical analysis and the validation of these candidate genes via complementation.

MATERIAL AND METHODS

Bristol Research and Development approval

Consent to acquire the microbiological samples from the environment and staff within the North Bristol NHS Trust was subject to approval from their Research and Innovation Department. This required a detailed Project Proposal approved by their Infection Control team, the completion of an Integrated Research Application System (IRAS) form, separate Site-Specific Investigation (SSI) forms for both hospitals involved and participation information sheets for potential HCW participants to be assessed for ethical approval. In addition to this, a Research Passport was required for access to the NHS sites. In total this process took about 6 months due to the number of parties involved which needed to approve the various aspects of the project.

Bristol sample collection

Using sterile swabs stored in charcoal Amies transport medium (MWE), general microbiological samples were taken from 118 separate fomites/surfaces at the Barbara Russell Children's Burns Unit, in Frenchay Hospital (Bristol) 3 months prior to the units relocation. A further 36 samples were taken from rooms associated with the Frenchay Hospital Surgical Theatre. The surgical equipment swabbed were swabbed before surgery, after surgery with a patient that presented with necrotising fasciitis (after surface cleaning with chlorine based cleaning products) and once more after a process called 'fogging', in which a vaporised cleaning solution is pumped into the theatre. The surface area of items swabbed was roughly kept to $1m^2$, or, in the case of items smaller than that, the entire surface area available. Additionally, staff members were asked to, anonymously, take swabs of the entire surface area of their right hand, their right nasal cavity and the front and back of their ID cards. A total of 5 doctors, 8 nurses and 4 therapy staff participated in the study. Finally, the new, cleaned, empty ward site, prior to the move, was swabbed as before, in order to gain an insight into whether any pathogens that presented a challenge after the move were introduced as a result of the move.

Growth conditions for bacterial isolates from Bristol

Mixed swab samples taken from the Bristol Children's Burns were streaked onto both Tryptic Soy Agar (TSA) plates (Thermo Scientific). All growth for each item swabbed were collectively picked and grown overnight at 37^oC (shaken) in 15ml of Tryptic Soy Broth (TSB), then a 1ml

aliquot of the inoculum was stored in a 20% glycerol in TSB solution at -80^{o}C for further analysis at a later date.

Verification of species diversity present within Bristol glycerol stocks

In order to verify the efficacy of the isolation strategy used, a sub-section of the mixed-species glycerol stocks acquired (all 69 swabs taken from the new unit) was streaked onto three separate varieties of selective media; Mannitol Salt Agar (for *Staphylococcus spp.* isolation), Pseudomonas Isolation Agar and Bile Esculin Agar (for *Enterococococcus spp.* isolation) and incubated overnight at 37^{o}C for 18hrs, after which point the number of colonies per well were counted and compared.

Bacterial propagation of ST228

From 20% (v/v) glycerol stocks, 207 isolates of ST228 (taken from the University Hospital of Lausanne), 95 isolates of ST239 (taken from four separate hospitals in Turkey (Marmara, Istanbul, Dokuz Eylul and Hacettepe University Hospitals)), and 31 selected transposon mutants (see below) were plated onto TSA (Sigma-Aldrich) and incubated for 18hr at 37^{o}C. Single colonies were then picked and inoculants propagated in 5ml aliquots of sterile TSB (Sigma-Aldrich) at 37^{o}C for 18hr at 120rpm in glass universal tubes.

Measuring δ toxin and PSM alpha -1, -2 and -3 through vesicle of phospholipid vesicles

Vesicle suspensions were made from combining 75µl 25 mol % 10,12-Tricosadiynoic acid (TCDA), 159µl 53 mol % 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 6µl 2 mol % 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPTE) and 60µl 20 mol % cholesterol (suspended in chloroform). Nitrogen gas was then used to dissolve the cholesterol from the fatty acid components, which were then rehydrated with 10ml of 50mM 5(6)-carboxyfluorescein (CF) in HEPES buffer and vortexed. A three times freeze/thaw cycle was then carried out, with the lipid suspension heated at 75^{o}C for 10 min then immersed in liquid nitrogen for 10 min. The solution was then homogenised at room temperature for 30 min and extruded three times at 50^{o}C through 2 100nm filter discs. The vesicles, that had encapsulated the CF fluorescent dye, were then purified through an Illustra Nap-25 column (GE healthcare) and, after 24 hrs, cross-linked with a CL1000 Ultraviolet crosslinker for 6 secs.

In triplicate Nunc 96-well plates, 160µl of the diluted inoculum of four separate ST228 isolates (isolated from patient blood cultures from the years 2000, 2001, 2006 and 2009 respectively), was added to 40µl of the lipid vesicle solution. The samples were then grown at 37^{o}C for 20

hrs, and fluorescence was measured at 485-12, 520em every 15min. 0.01% v/v Triton X-100 was also analysed as a positive control while HEPES buffer was used as a negative control. The *E. coli* strain DH5α was also analysed for comparison.

Assessment of phage susceptibility of ST228 isolates

Phage K and Dra88, isolated by Diana Alves, were collected from crude sewage and enriched with various S. aureus strains. This was carried out through added 5ml of chloroform for every 1l of crude sewage, leaving for 1 hr, then removing the resultant top layer of liquid. To this, 5ml of an overnight culture of S. aureus (incubated at 37^{o}C in TSB), 1 mM MgSO4 and 1mM CaCl2 were added and the mixture made up to 500ml with TSB and incubated at 37^{o}C overnight. A 10ml aliquot of this inoculum was added to 1M NaCl (in chloroform) and the culture centrifuged for 30 min at 4000xg. The resultant supernatant (containing the phage) was then filter sterilized with a 0.22µm Millipore filter and stored at 4^{o}C. Phage titer at 10^{8} pfu/ml were collected and diluted by SM buffer to 106, 104 and 102 pfu/mls and spotted onto a TSA plate containing 30ml of top agar (12g TSB to 2.6g TSA) and overnight cultures of ST228 isolated during 2001, 2006 and 2009 (in TSB, grown while shaken at 37^{o}C). The plates were then incubated overnight at 37^{o}C, at which point plaques within the resulting bacterial lawn were analysed.

ST239 and transposon mutant propagation

From 20% (v/v) glycerol stocks, 95 isolates of ST239, taken from four separate hospitals in Turkey (Marmara, Istanbul, Dokuz Eylul and Hacettepe University Hospitals), and 31 selected transposon mutants (see below) were plated onto TSA and incubated for 18hr at 37^{o}C. Single colonies were then picked and inoculants propagated in 5ml aliquots of sterile TSB at 37^{o}C for 18hr at 120rpm in glass universal tubes in order to maintain optimal exogenous protein secretion.

Quantifying biofilm forming potential using crystal violet staining for ST228 isolates

In triplicate Nunc 96-well plates, 200µl of the 31 ST228 isolates taken from 2000-2009 was deposited in a well in each plate and grown overnight at 37^{o}C. Excess media was then tipped out and the plates were washed three times with 200µl sterile Phosphate Buffer Solution (PBS). The plates were then dried at 80^{o}C for 1 hr and stained with 0.1% crystal violet solution for 15 min. The stain was washed off three time with 200µl PBS and the resulting stained biofilm was then dissolved with 200µl 100% ethanol and OD measurements were made for

each sample at 595nm. Strain JE2 of USA300 was also analysed for comparison while PA01 and TSB were included as positive and negative controls respectively.

Quantifying the general production of proteases in ST239 supernatants via a milk plate assay

Concentrated TSA (x2 concentrated, 15g in 400ml) and 100ml 50% skimmed milk solution (Sigma-Aldrich, Dorset, UK) were autoclaved separately, then combined and poured into plates. Once resulting TSA with 10% milk plates had set, they were dried for an additional 15min. Then, in aseptic conditions, 1cm diameter wells were punched into the agar. Bacterial supernatants were obtained (in triplicate) through transferring 1ml aliquots of the overnight cultures of interest into sterile 1.5ml eppendorf tubes, then centrifuged twice for 15min at 14000rpm, to ensure that all free-floating cells are removed from the supernatant. Aliquots of 50µl of supernatant were then removed and pipetted into separate wells in the milk plates. The plates were then incubated for 18hr at 37^{o}C. Zones of clearance as a result of casein hydrolysis within the agar were then measured to the nearest 0.25mm using a ruler.

Modified crystal violet staining as to quantify ST239 and transposon mutant biofilm formation

In sterile 96-well plates, with the outermost wells filled with 200µl of water, 100µl of TSB with 0.5% glucose (Sigma-Aldrich, Dorset, UK) filter sterilised through 0.22µm filters were dispensed into each well. Triplicate 2.5µl aliquots of the overnight cultures were then added to the wells and, in high moisture conditions the plates were incubated for 24hr at 37^{o}C. The media and any planktonic bacteria was then carefully discarded and the biofilm that formed at the bottom of the well was washed four times with 250µl sterile PBS. Then 150 µl of 1% crystal violet solution was added to each well and left for 30min at room temperature. The wells were again washed four times with PBS and the stained biofilm then resuspended in 200µl 7% acetic acid. OD was then measured at 595nm. This was repeated in triplicate with three different inoculants per isolate.

Selection of appropriate transposon mutants in genes significantly associated with protease production and biofilm formation

Using previously obtained sequence data, a quantitative association study was conducted on the ST239 isolates to identify SNPs that were significantly associated with protease production and biofilm formation using PLINK (http://pngu.mgh.harvard.edu/purcell/plink)(Purcell *et al.*, 2007). SNPs that produced a Bonferroni adjusted P-value <10^{-5} were selected as potentially affecting candidate genes. SNP loci in the ST239 reference TW20 (accession number FN433596) were then mapped against a

USA300_FPR3757 (accession number NC_007793.1) background, as this was the background of the transposon library available. The means through which these transposon mutants were produced is described elsewhere (Fey *et al.*, 2013). The loci positions of regions that showed sequence homology of over 98% in USA300_FPR3757 were then inserted into the University of Nebraska Medical Centre's functional genomics explorer (http://app1.unmc.edu/fgx/index.html) and transposon mutants in the collection with a transposon insertion in the gene affected by the SNP of interest in the ST239 background were selected for further analysis. Any SNP within an intergenic region in USA300_FPR3757 not present in the strain collection, or located in a loci in the ST239 background with no homologous loci in USA300_FPR3757, or within a pathogenicity island present in ST239 but not found in USA300_FPR3757 were omitted.

Production of chemically competent DH5α

DH5α was propagated for 18hrs before being inoculated in LB (1:1000). The culture was then incubated at 37°C at 180 rpm for 2-4hrs to ensure an OD600 of 0.5-0.7. Cells were then chilled on ice for 30mins before being centrifuged for 10mins at 4°C (3000 rpm) and washed with ice-cold deionised water. This was then centrifuged for a further 10mins at 4°C and resuspended in 15ml of chilled, sterile 0.1M $CaCl_2$. These cells remained on ice for 1-2hrs before being centrifuged again for 10mins at 4°C. The resulting pellets were resuspended in 500µl ice-cold, sterile 0.1M $CaCl_2$ containing 15% glycerol.

Molecular cloning of wild type biofilm candidate genes into their respective transposon mutants

Primers were designed with selected restriction sites in order to amplify loci of interest generated from GWAS of biofilm formation in ST239 (Appendix A) (Eurofins). These primers were diluted with sterile, deionized water to bring the concentration of primer to 200pmol/ml. Using the Phusion HF/GC DNA polymerase as instructed via their protocol (NEB), with the following thermocycler parameters; a) initial denaturation (98^{o}C, 30 secs), b) denaturization (98^{o}C, 10 secs), c) annealing stage (62^{o}C (*ccrB*, *sgaT* and *hyp*) or 59^{o}C (*aap* and *mecA*), 30 secs), d) elongation stage (72^{o}C, 30 secs/kb, b-d repeated for 30 cycles) e) final elongation (72^{o}C, 10 secs) and f) storage (4^{o}C). PCR product was then purified using the GeneJET PCR purification kit (Thermo) and the resulting DNA yield measured using the Qubit DNA concentration kit (Thermo). Successful amplification was verified by running samples on a 1% agarose gel (85V, 30 mins).

All primers were either digested with *Eco*RI-HF (forward strands) or KpnI-HF (reverse strands) (NEB), with purified pRMC2 also digested with the same restriction endonucleases separately. In accordance with the CutSmart buffer (NEB) protocol, forward and reverse strands were digested concurrently in the same reaction. All restriction digests were incubated at 37^{o}C for 30 mins. The pRMC2 backbone and digested primer pairs (one for each candidate gene) were then ligated using the T4 DNA ligase protocol (NEB), with the reaction incubated at 37^{o}C for 10 mins, then heat inactivated at 65^{o}C for 10 mins before being chilled on ice. Aliquots of the ligation products were checked to ensure insertion of candidate genes through electrophoresis in a 1% agarose gel (85V, 30 mins).

Chemically competent DH5α aliquots of 50µl were incubated on ice with 5µl aliquots of each ligation solution for 30 mins, before heat-shocking at 42^{o}C and then placing on ice for 5 mins. The DH5α and plasmid mixtures were then added to 750µl of LB broth and incubated at 37^{o}C for 1hr. This was centrifuged for 1min at room temperature (3000rpm) and the supernatant mostly discarded: any remaining supernatant was uses to resuspend the pellet. This was then plated out onto LB ampicillin plates at incubated for 18hrs at 37^{o}C. From single colonies, the transformed DH5α were then propagated for 18hrs at 37^{o}C, from which the pRMC2-backbones containing the candidate genes of interest were extracted used the GeneJet Midi Kit (Thermo).

Purified plasmids were then first electroporated into RN4220, then in the relevant transposon mutant. In both instances, the staphylococcal isolates were grown for 18hrs at 37^{o}C, and 50µl aliquots of these overnight cultures were added to BHI and propagated at 37^{o}C until an OD_{550} of 0.2-0.25 is reached. These cells were washed 4 times with 3ml of filter sterilized, ice-cold 0.5M sucrose, centrifuging at 4^{o}C (3000rpm) for 10 minutes each time. After the final wash the resultant pellet was resuspended in 100µl of 0.5M sucrose solution. An aliquot of 100µl of these cells was missed with 50µl of purified plasmid and incubated on ice for 20 mins before electroporation using 0.2cm sterile electroporation cuvettes (Molecular BioProducts) and a Micro Pulser (Bio-Rad) set on their preinstalled staphylococcus settings. These cells were then used to inoculate 750µl of BHI before being incubated at 37^{o}C for 18hrs before aliquots were plated on TSA containing 10 µg/ml chloramphenicol for 18hrs at 37^{o}C.

CHAPTER 1: SURVEYING CHANGES TO THE MICROBIAL BURDEN OF A BURN'S UNIT CAUSED BY ITS RE-LOCATION

INTRODUCTION

Hospital-acquired infections represent a significant burden to healthcare services, with healthcare-associated infections acting as a significant cause of morbidity and mortality globally. Media coverage of the rise in rates of mortality as a consequence of the various healthcare-associated pathogens that have emerged over the year has resulted in an increased awareness in the general public of the challenges faced in combating the emergence and transmission of pathogens in hospitals. The diversity of pathogens that can cause these infections, in addition to the plethora of ways in which these pathogens colonise the environment and evade the various strategies employed to treat their infections have made efficient infection control measures of paramount importance to healthcare providers internationally.

Historically, various studies have highlighted the importance of various practices that can be employed by HCWs to reduce the spread of infection from one patient to another, such as maintaining a high standard of hand-hygiene, implementing compulsory reporting of infections and avoiding over-crowding where possible (Eames *et al.*, 2009). However, the increased availability of pathogen sequence data and robust software for their analysis has resulted in a paradigm shift where the abundance of new insights into the transmission of pathogens from one patient to another may pave the way for a variety of new preventative strategies and infection treatments, like the changes in hospital infection control policy that resulted in the identification of colonisation 'hot-spots' in the hospital environment leading to the targeted cleaning efforts used in recent years (Narui *et al.*, 2008). However, while much of the literature consists of phylogenetic analysis of pathogenic outbreaks that altered the landscape of already established bacterial flora, there have been a comparatively modest number of studies that have focused on the actual development of an established environmental microbiome in hospital settings. Of the few that have, however, a large proportion of these studies have done so through investigating the consequences of the geographical relocation of discrete clinical units.

The first paper to study the transmission of nosocomial pathogens as the result of the movement of medical equipment and personnel into another building focused on the University of Wisconsin Hospital's move from a 56-year-old building to a newly-built, larger facility, with samples being taken from fomites, work surfaces and the air before and after the move. Prior to patient occupancy, significantly fewer common nosocomial pathogens were isolated from the hospital environment directly after it's relocation. However, pathogenic species abundance and diversity both returned to pre-relocation levels 12 months after the move. *Acinetobacter spp*. and Pseudomonads were the most frequently isolated genera of bacteria in these screens. Interestingly, *Staphylococcus aureus*, although also commonly isolated, was seen to be just as prevalent in the new location, directly after the move, as the old location. This seminal paper reached the conclusion, therefore, that levels of bacterial contamination of a clinical environment had a negligible relationship to the incidence of post-admission infection (Maki *et al.*, 1982).

A more recent study, however, investigated potential changes in MRSA transmission after the relocation of the Brooke Army Medical Centre to a purpose-built facility with a higher degree of bed segregation and one sink assigned to each room. This study involved measuring patient nasal colonisation by MRSA in both locations, alongside a retrospective analysis of all MRSA isolated from routine patient screens for 2 years prior to the move, and again 2 years after. Hand-hygiene compliance by healthcare workers (HCWs) was also monitored. In a similar finding to the previous study, they revealed that the rate of MRSA transmission did not change significantly after the medical centre's relocation. This was, in part, attributed to the fact that rates of transmission were already deemed to be rather low, and the authors suggested that a more meaningful conclusion on the effects of increased patient isolation and a higher sink-to-bed ratio could be gleaned from a similar study carried out by a ward with a higher initial pre-move incidence of MRSA transmission (Vietri *et al.*, 2004).

Changes in MRSA prevalence before and after the relocation of a Chinese intensive care unit (ICU) from a 6-bed ward to a new 20-bed ward was carried out in 2014. Isolates of MRSA were acquired from both skin-and-soft-tissue swab samples, as well as from clinical specimens, from the patients immediate surrounding and from HCWs. In this study a significant drop of MRSA-positive SST swabs on admission was seen in the new ward in comparison to the old one. However, a 3-fold increase in new cases per imported MRSA case was observed in the new

ward, suggesting that, in the relatively sanitised new environment, MRSA transmission increased. However, these findings were largely attributed to a reduced level of HCW hand hygiene compliance after patient contact (Chen *et al.*, 2014).

Given the relative lack of comprehensive investigations in changes in transmission rates of clinically relevant pathogens after the relocation of a clinical environment to a new building, likely owing to the infrequency of these relocations, a unique opportunity to study transmission dynamics after the move of a paediatric burn's unit presented itself in 2014. The Barbara Russell Children's Burns Unit was moved from its old site to a deep-cleaned, larger unit in a neighbouring hospital, providing a rare opportunity to explore what effect the relocation of potentially contaminated equipment and colonised medical personnel would have on the dissemination of pathogens in this new environment, as well as investigating the extent to which, if at all, species diversity is restored to levels seen in the old site, given that incidences such as these may provide novel insights into the extent to which transmission bottlenecks influence the phylodynamics of niche healthcare environments.

This chapter focuses on an attempt to use a metagenomic approach to looking at changes in population dynamics of a range of potential pathogens, both before and after the move on a metagenomics level, in which mixed-species genomic DNA samples from individual surfaces and HCWs were to be sequenced in a manner sensitive enough to identify relatively sparse populations of bacteria and provide a snap-shot of the percentage abundance of various species on a particular surface in a given time point, Analysis of the resultant sequence data would utilise PathoScope 2.0, due to its increased scope, speed and accuracy in comparison to other bioinformatics frameworks used for metagenomics data at the time (Hong *et al.*, 2014).

RESULTS

Sample collection

Using sterile swabs stored in charcoal Amies transport medium (MWE), general microbiological samples were taken from 118 separate fomites/surfaces from 20 different rooms within the Barbara Russell Children's Burns Unit (Figure 4), in Frenchay Hospital (Bristol) 3 months prior to the unit's relocation. The surface area of items swabbed was roughly kept to $1m^2$, or, in the case of items smaller than that, the entire surface area available.

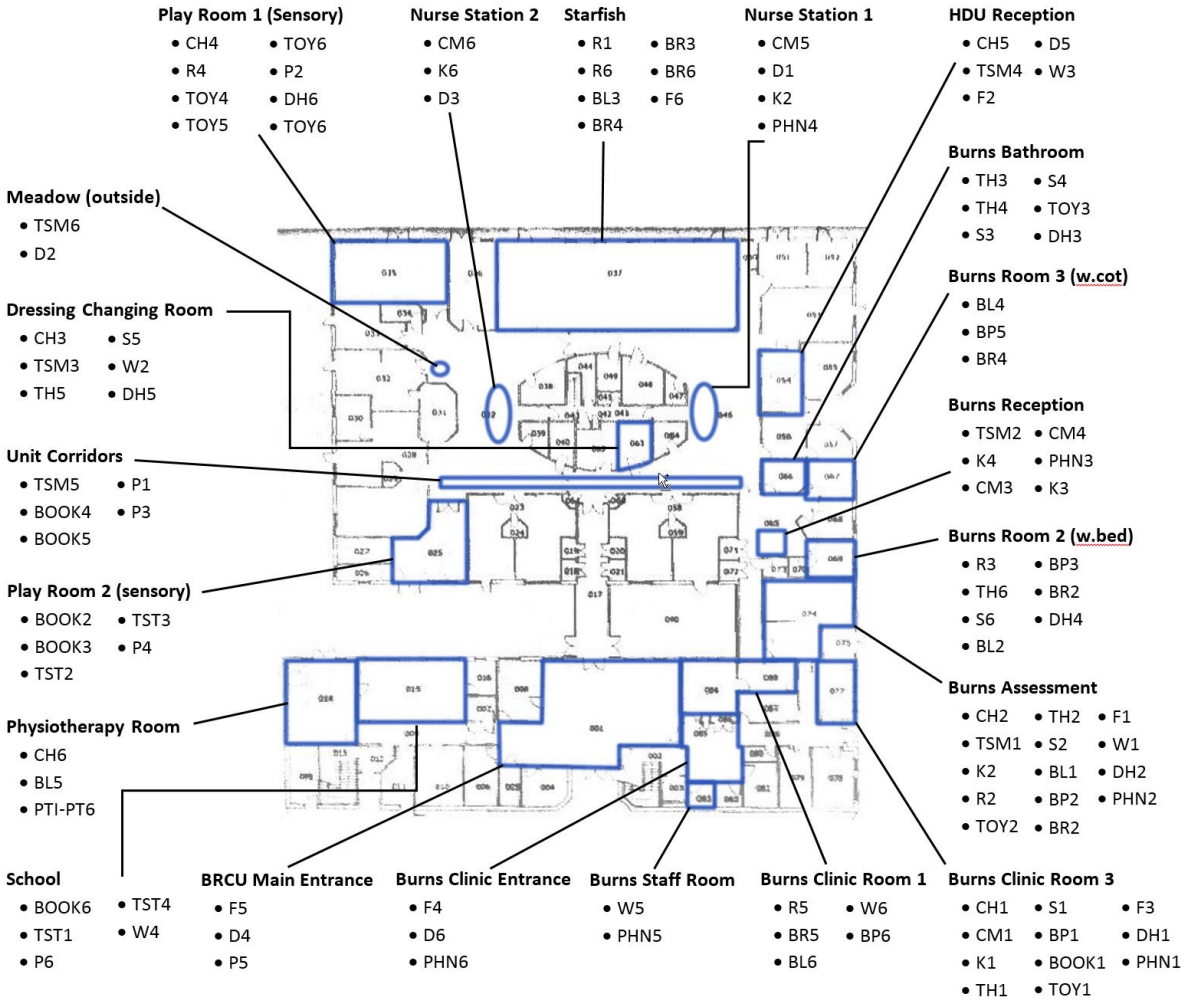

Figure 4 - Floor plan of Barbara Russell Children's Burns Unit, with rooms swabbed highlighted and the reference IDs of the samples taken listed (See appendix for key)

Additionally, prior to the relocation of the hospital, staff members were asked to, anonymously, take swabs of the entire surface area of their right hand, their right nasal cavity and the front and back of their ID cards. A total of 5 doctors, 8 nurses and 4 therapy staff participated in the sampling prior to the ward relocation.

Prior to the move, environmental swabs were also taken from the new, cleaned, empty ward site, as described previously, in order to gain an insight into the indigenous bacterial flora of the site initially. A total of 22 swabs were taken, from surfaces in the new ward (as no equipment had been transferred at the time) (Figure 7). All isolates thereafter were

propagated and stored as advised for simultaneous pooled-sequencing. Swabs were not taken of the ward as anticipated 3 months after the move due to a preliminary survey of the microbial diversity of the samples taken previously showing a shift in microbial diversity as a result of the culturing technique used (

Table 1).

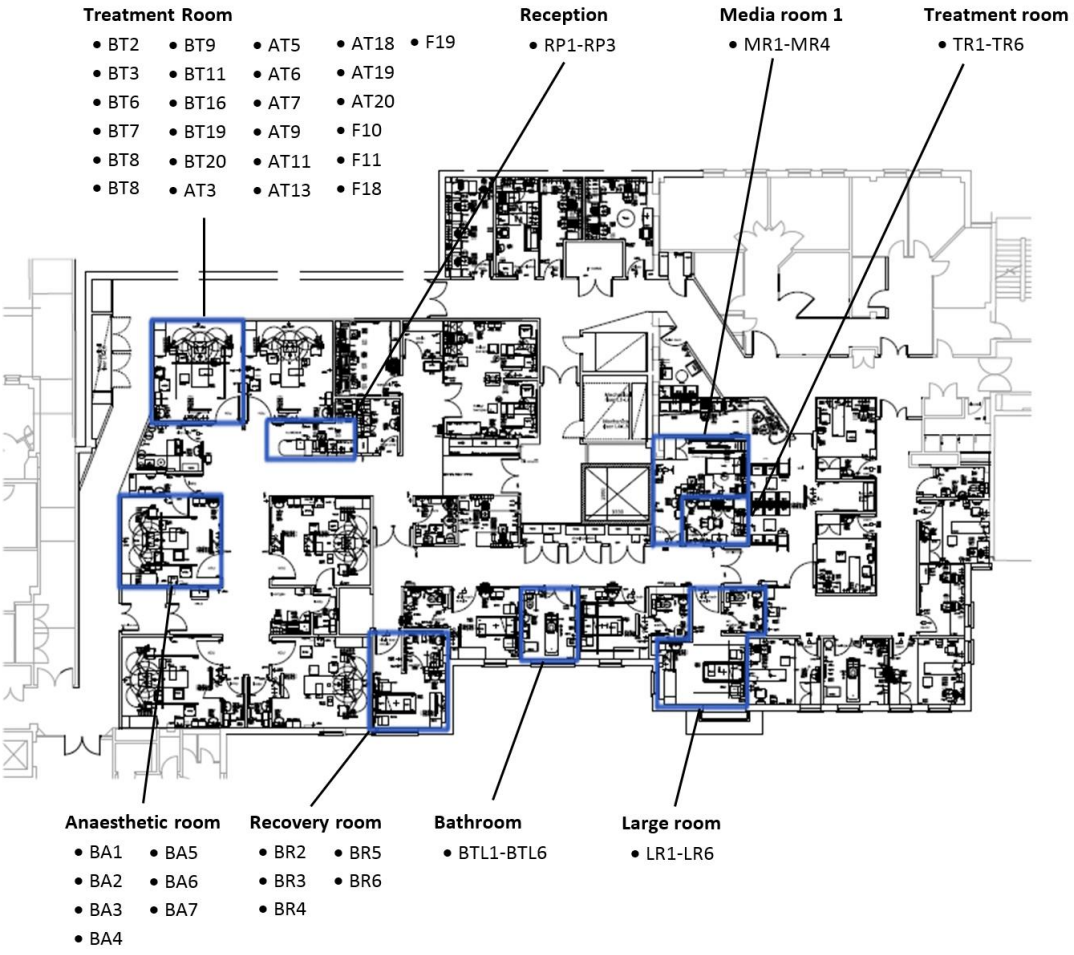

Figure 5 - Floor plan of the Bristol Royal Children's Hospital, with swabbed rooms highlighted and reference IDs shown

PRELIMINARY SURVEY OF MICROBIAL DIVERSITY IN EACH SAMPLE

Although advised to culture each swab prior to storage, a concern was raised that this step may have eliminated (or significantly altered) the diversity present in each swab. Given the expense associated with DNA extractions and sequencing of each swab, a preliminary attempt to determine whether a single species had out competed the others during the culturing stage of the storage process was undertaken using selective agar using staphylococci, pseudomonas and enterococci as proxy for overall species diversity.

Table 1 Growth of Bristol environmental and staff swabs on media selective for Staphylococci (MSA), Pseudomonas (PIA) and Enterococci (BEA)

	Growth on selection agar from samples collected before the move		
Sample	***Mannitol Salt Agar***	***Pseudomonas Isolation Agar***	***Bile Esculin Agar***
BRCU1-CH1	No growth	No growth	Lawn
BRCU1-CH2	6-25 colonies	No growth	Lawn
BRCU1-CH3	1-5 colonies	No growth	Lawn
BRCU1-TSM1	No growth	No growth	Lawn
BRCU1-TSM2	6-25 colonies	No growth	Lawn
BRCU1-TSM4	26-50 colonies	No growth	Lawn
BRCU1-CM1	1-5 colonies	No growth	Lawn
BRCU1-CM2	1-5 colonies	No growth	Lawn
BRCU1-CM4	No growth	No growth	Lawn
BRCU1-K1	6-25 colonies	No growth	50+ colonies
BRCU1-K2	6-25 colonies	No growth	Lawn
BRCU1-K3	1-5 colonies	No growth	Lawn
BRCU1-K4	1-5 colonies	No growth	Lawn
BRCU1-K5	No growth	No growth	50+ colonies
BRCU1-K6	26-50 colonies	No growth	Lawn
BRCU1-R1	6-25 colonies	No growth	Lawn
BRCU1-R2	1-5 colonies	No growth	Lawn
BRCU1-R4	1-5 colonies	No growth	Lawn
BRCU1-TH1	1-5 colonies	No growth	Lawn
BRCU1-TH3	6-25 colonies	No growth	Lawn
BRCU1-TH4	6-25 colonies	No growth	Lawn
BRCU1-S3	1-5 colonies	No growth	Lawn
BRCU1-S4	1-5 colonies	No growth	Lawn
BRCU1-S5	1-5 colonies	No growth	Lawn
BRCU1-BL2	6-25 colonies	No growth	Lawn
BRCU1-BL4	1-5 colonies	No growth	Lawn
BRCU1-BL5	26-50 colonies	No growth	Lawn
BRCU1-BP1	1-5 colonies	No growth	Lawn

BRCU1-BP4	26-50 colonies	No growth	Lawn
BRCU1-BP6	6-25 colonies	No growth	Lawn
BRCU1-PT1	1-5 colonies	No growth	Lawn
BRCU1-PT4	6-25 colonies	No growth	No growth
BRCU1-PT5	6-25 colonies	No growth	Lawn
BRCU1-Book3	1-5 colonies	No growth	No growth
BRCU1-Book4	6-25 colonies	No growth	No growth
BRCU1-Book5	No growth	No growth	No growth
BRCU1-Toy1	No growth	No growth	Lawn
BRCU1-Toy2	1-5 colonies	No growth	Lawn
BRCU1-Toy3	6-25 colonies	No growth	Lawn
BRCU1-Toy4	1-5 colonies	No growth	Lawn
BRCU1-Toy5	1-5 colonies	No growth	Lawn
BRCU1-TST1	6-25 colonies	No growth	Lawn
BRCU1-TST2	1-5 colonies	No growth	Lawn
BRCU1-TST3	6-25 colonies	No growth	Lawn
BRCU1-TST4	1-5 colonies	No growth	Lawn
BRCU1-D3	1-5 colonies	No growth	No growth
BRCU1-D4	1-5 colonies	No growth	No growth
BRCU1-D5	No growth	No growth	No growth
BRCU1-W1	1-5 colonies	No growth	No growth
BRCU1-W2	No growth	No growth	Lawn
BRCU1-W3	No growth	No growth	Lawn
BRCU1-W4	1-5 colonies	No growth	Lawn
BRCU1-W5	6-25 colonies	No growth	Lawn
BRCU1-W6	6-25 colonies	No growth	Lawn
BRCU1-P1	No growth	No growth	Lawn
BRCU1-P2	6-25 colonies	No growth	Lawn
BRCU1-P3	Lawn	No growth	Lawn
BRCU1-P4	1-5 colonies	No growth	Lawn
BRCU1-P5	6-25 colonies	No growth	Lawn
BRCU1-P6	6-25 colonies	No growth	Lawn
BRCU1-DH1	6-25 colonies	No growth	Lawn
BRCU1-DH3	No growth	No growth	Lawn
BRCU1-DH4	Lawn	No growth	Lawn
BRCU1-Phn1	No growth	No growth	Lawn
BRCU1-Phn2	1-5 colonies	No growth	Lawn
BRCU1-Phn3	Lawn	No growth	Lawn
BRCU1-Phn4	6-25 colonies	No growth	50+ colonies
BRCU1-Phn5	6-25 colonies	No growth	Lawn
BRCU1-Phn6	1-5 colonies	No growth	Lawn

	Growth on selection agar from samples collected from hospital staff		
Sample	***Mannitol Salt Agar***	***Pseudomonas Isolation Agar***	***Bile Esculin Agar***
N1H	26-50 colonies	No growth	Lawn
N1N	6-25 colonies	No growth	No growth
N1C	1-5 colonies	No growth	Lawn
N2H	26-50 colonies	No growth	Lawn
N2N	1-5 colonies	No growth	No growth
N2C	6-25 colonies	No growth	Lawn
N4H	6-25 colonies	No growth	No growth
N4N	26-50 colonies	1-5 colonies	No growth
N4C	1-5 colonies	No growth	Lawn
N5H	1-5 colonies	26-50 colonies	No growth
N5N	1-5 colonies	No growth	Lawn
N5C	No growth	No growth	Lawn
N6H	No growth	6-25 colonies	6-25 colonies
N6N	6-25 colonies	No growth	Lawn
N6C	Lawn	1-5 colonies	1-5 colonies
N7H	6-25 colonies	No growth	Lawn
N7N	1-5 colonies	No growth	51+ colonies
N7C	1-5 colonies	No growth	Lawn
N8H	Lawn	No growth	Lawn
N8N	1-5 colonies	No growth	Lawn
N8C	No growth	No growth	Lawn
N9H	6-25 colonies	1-5 colonies	Lawn
N9N	No growth	1-5 colonies	No growth
N9C	1-5 colonies	No growth	Lawn
N10H	6-25 colonies	No growth	No growth
N10N	Lawn	No growth	No growth
N10C	6-25 colonies	No growth	No growth
D2N	26-50 colonies	No growth	No growth
D3H	6-25 colonies	No growth	Lawn
D3N	Lawn	No growth	No growth
D7H	No growth	No growth	No growth
D7N	6-25 colonies	No growth	Lawn
D7C	No growth	No growth	6-25 colonies
D8H	26-50 colonies	No growth	No growth
D8N	6-25 colonies	No growth	26-50 colonies
D8C	26-50 colonies	No growth	6-25 colonies
D9H	No growth	No growth	26-50 colonies

D9N	6-25 colonies	No growth	1-5 colonies
D9C	26-50 colonies	No growth	Lawn
H1H	No growth	No growth	No growth
H1N	1-5 colonies	No growth	No growth
H1C	6-25 colonies	No growth	No growth
H3H	1-5 colonies	No growth	No growth
H3N	1-5 colonies	No growth	No growth
H3C	1-5 colonies	No growth	Lawn
H7H	1-5 colonies	No growth	No growth
H7N	26-50 colonies	No growth	No growth
H7C	6-25 colonies	No growth	1-5 colonies
H8H	6-25 colonies	No growth	Lawn
H8N	6-25 colonies	No growth	51+ colonies
H8C	1-5 colonies	No growth	No growth

No growth of pseudomonas, following the chosen method of culturing the collected swabs in TSB, was seen in any of the 69 culture samples tested, despite the fact that *Pseudomonas aeruginosa* is a relatively common hospital-acquired opportunistic pathogen, known to colonise clinical environments readily (Fazeli *et al.*, 2012). Additionally, the growth of enterococcal species consistently and dramatically dwarfed the levels of growth seen by staphylococci isolated from the same area.

Discussion and Conclusions

In hindsight, the use of TSB, a selective medium, in a project largely dependent on the accurate and sensitive determination of the species diversity picked up in a single swab, was an error. The likelihood of the samples collected containing a sufficient degree of species diversity to accurately study changes in the population dynamics of healthcare-associated pathogens before and after the move of this ward was therefore too low to warrant the resources needed to take the project further, given that a second collection of the burns' unit prior to the move is no longer possible.

In this instance, while it would not have been appropriate to have carried the project on, the high incidence of nosocomial infection in paediatric burns' patients warrants investigation into the potentially unique transmission chains that represent such a significant burden currently. Nosocomial transmission of pathogens to burn wounds have historically been largely

associated with direct contact of the burn to the colonised hands of healthcare workers (Church *et al.*, 2006). And while poor infection control practices do increase the risk of nosocomial infection, good hygiene practices sadly are not enough to prevent MRSA transmission from staff to patients. The increase of MRSA carriage caused by close contact to patients is of particular concern in a burns unit, since patients are regularly in close contact with staff for wound debridement and dressing changes (Albrich and Harbarth, 2008). Therefore, determining effective treatment strategies through increasing our understanding of the population dynamics of common pathogens is of paramount importance.

Should another opportunity arise to study changes in pathogen transmission and population dynamics as a consequence of a ward or hospital's physical relocation to a new site, the potential novelty and utility of using a similar pooled-sequencing approach to the resultant metagenomics analysis should not be discounted.

CHAPTER 2: IDENTIFYING PHENOTYPIC DIFFERENCES ASSOCIATED WITH A *S. AUREUS* OUTBREAK IN A TERTIARY CARE HOSPITAL.

THE LAUSANNE ST228 COLLECTION

The MRSA strain ST228, also known as the South German Epidemic strain or the Italian clone, is a multiply antibiotic resistant genotype belonging to the clonal complex (CC) 5, which, along with CC8, are the most prevalent CCs worldwide. ST228 is largely prevalent in central European countries, including Germany, Italy, Austria and Switzerland (Monecke *et al.*, 2009) (Figure 6).

Figure 6 - The prevalence of the MRSA clone ST228 within central Europe (Dominique S Blanc; personal communication).

ST228-MRSA-I was first detected in Switzerland in the late 90s and its spread has been restricted between Geneva and Lausanne tertiary care hospitals. In addition to this strains geographical isolation, within the tertiary care hospital of Lausanne, this clone became responsible for two outbreaks during 2001 and late 2003/early 2004. The prevalence of this clone then diminished, at which point the hospital considered its infection control procedures to have been effective. However, as a precaution the hospital continued to implement these practices (Dominique Blanc, personal communication). Despite this, however, during late 2008, another epidemic was observed, with the strain disseminating at an unusual rate,

affecting over 500 patients within a year and a half, despite other clones remaining relatively dormant (Vogel *et al.*, 2012b) (Figure 9).

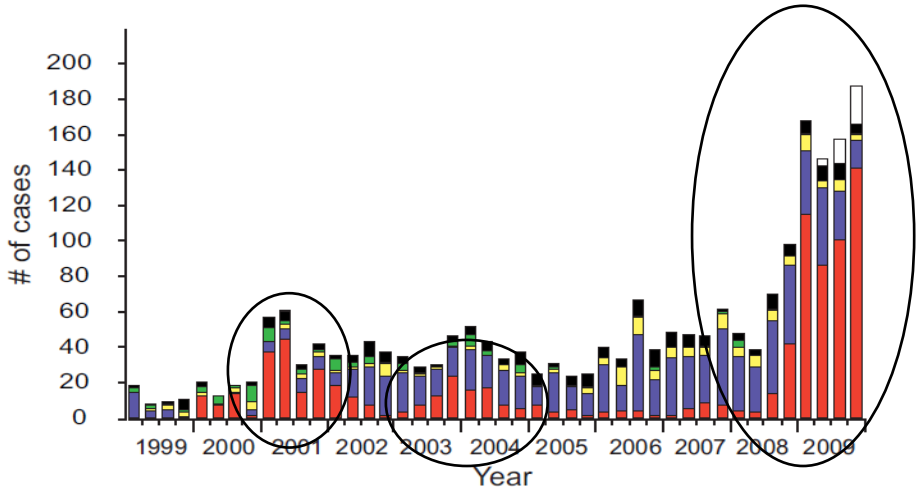

Figure 7 - The number of cases of patient with positive swabs for various sequence types of MRSA (ST228 in red) (Vogel et al., 2012b).

In a genomic analysis of eight isolates collected in 2001, 2006 and 2008, Vogel *et al.* (2012) determined that all of the isolates were genetically very closely related, with most variation consisting of just a few SNPs and INDELs (insertions and deletions of nucleotides). Whatever genetic differences were observed could not be linked to the sudden spreading success of the strain (Vogel *et al.*, 2012b). However, the group highlighted that this may not necessarily mean that the cause of the outbreak had no genetic basis. A plausible case can be made that changes in phenotype that may have resulted in the rapid dissemination of the clone in 2008 could be a result of a multitude of additional factors that could mask a genetic cause for the breakout (for instance changes in post-transcriptional regulation). Thus it was theorised that the cohort would benefit a study of phenotypic variation at different times of isolation, after which point any phenotypic differences between outbreak isolates and those isolated during non-outbreak years can be studied molecularly across the strain collection.

BIOFILM FORMATION

Given the propensity of *S. aureus* to persist in clinical environments through colonising medical equipment and surfaces, an analysis of the isolates taken at different year's ability to

form biofilms was carried out. A microtiter biofilm assay that utilised crystal violet staining as a means of measuring adherence to the base of a 96-well plate was performed (Figure 8).

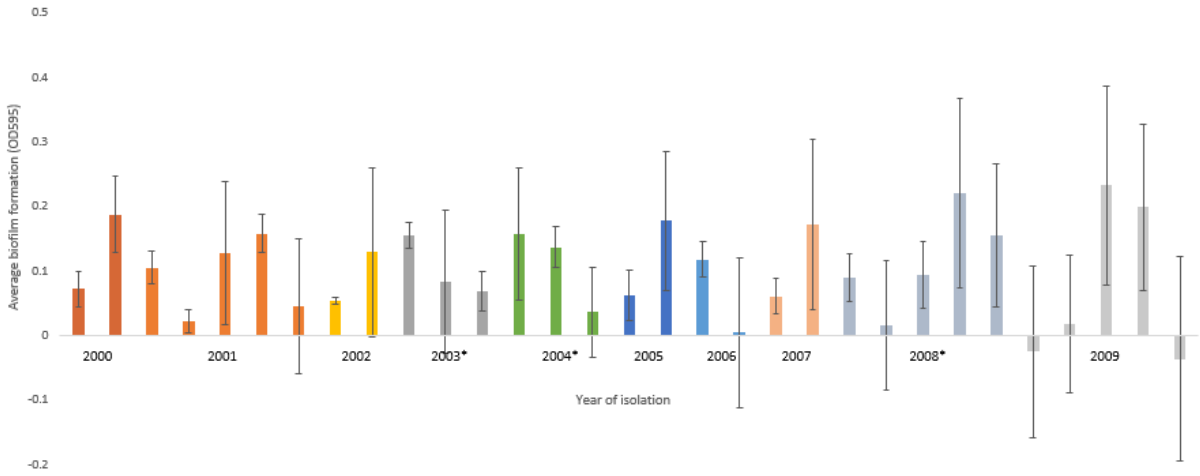

*Figure 8 - Biofilm formation of 31 ST228 isolates, as shown through relative retention of crystal violet, measured through OD595 (n=31), standardised against negative TSB control. * coincide with years in which a perceived outbreak of ST228 had occurred. Error bars represent standard deviation (SD)*

The biofilm data collected for the ST228 isolates were unusually low (view Fig. 20 as a point of comparison), given that the average biofilm production seen in low-biofilm producing clones are known to have OD_{595} of orders of magnitudes higher than is depicted in Figure 8. Additionally, the large standard deviation (relative to the OD figures reported) suggest little variation in the biofilm production of ST228 isolates throughout the years the hospital was under surveillance.

δ toxin and PSM alpha-1, -2 and -3 production in ST228

The *agr* operon acts as a population-density dependent quorum-sensing transcription modulator. In a recently developed high-throughput assay, it was discovered that the *agr* activity of stains of *S. aureus* could be analysed through the detection of the lysis of lipid vesicles encapsulating a fluorescent dye through the action of δ toxin and PSMs (-α, -β, -γ and –δ), two essential classes of toxin involved in MRSA virulence (Maisem Laabei *et al.*, 2014a). To gain an appreciation for the strains variation in toxin production the aforementioned

vesicle lysis test was used to assess the activity of the *agr* operon for the ST228 isolates and whether any changes in *agr* activity could be observed during the outbreak periods (Figure 9).

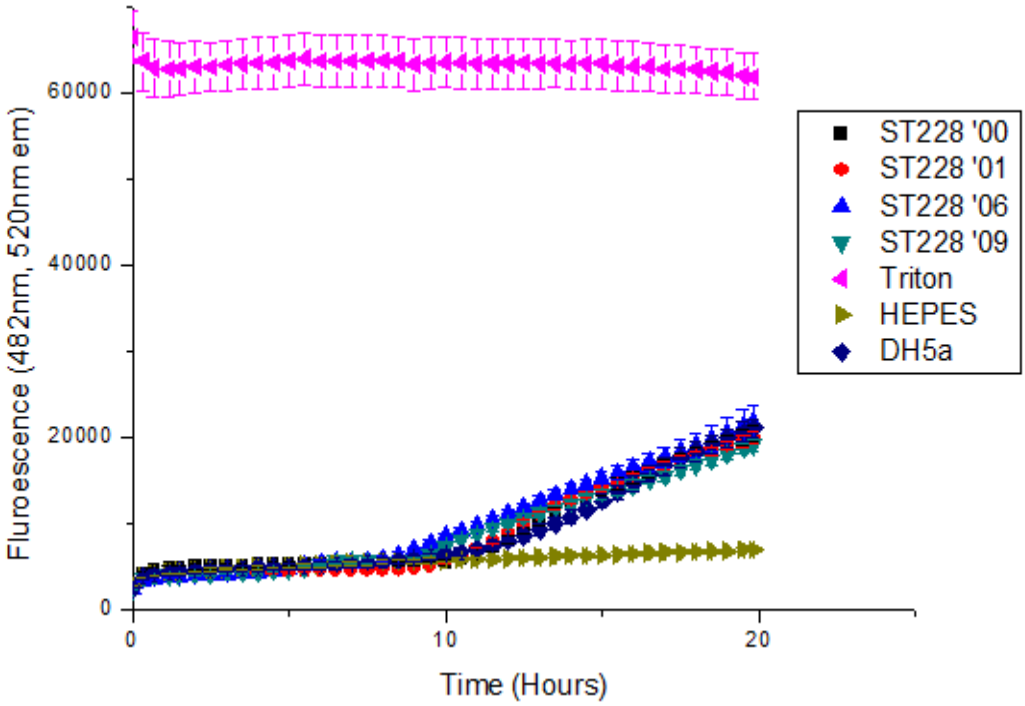

Figure 9 - Lipid vesicle lysis by ST228 isolates from 2000, 2001, 2006 and 2009, in comparison to Triton (positive control), HEPES buffer and DH5α (negative controls) (n=4. Error bars represent SD

ST228 displayed low production of δ toxin and PSMs, with levels of vesicle lysis comparable to the avirulent DH5α *E. coli* strain (Figure 9). No change in toxicity was observed between isolates taken from epidemic years and those taken from when ST228 was of less concern to the hospital.

Phage susceptibility of ST228

Given the number of bacteriophage that form part of the natural human microbiota, changes in phage susceptibility by a bacterial strain may result in a change in their ability to persist within a human host. With this, the susceptibility of ST228 to both Phage K and Dra88 (used due to their generality in terms of the number of host strains they are able to infect) was briefly assessed through spotting a solution containing these phages on a bacterial lawn of 3 ST228 isolates taken in 2001, 2006 and 2009 (with 2001 seeing a slight increase in the

incidence of infection, and 2009 being the year that the number of infections were highest), at various pfu/ml concentrations (Figure 10).

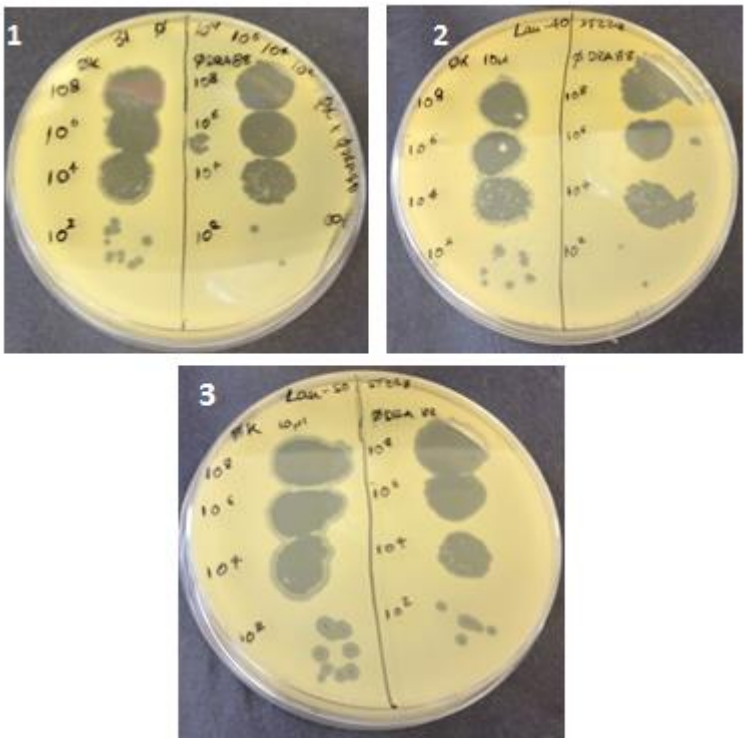

Figure 10 - Phage K (left of each plate) and Dra88 (right of each plate), at 108, 106, 104 and 102 pfu/ml, spotted onto a lawn of ST228, isolated at 2001 (1), 2006 (2) and 2009 (3)

All isolates tested showed a high degree of susceptibility to both phages tested, with cell lysis seen even at 10^2 pfu/ml (Figure 10). Following this, an analysis of the growth of these 3 ST228 isolates overnight was studied, with phage added 2 hours after the isolates initial incubation at 37^{o}C (Figure 11).

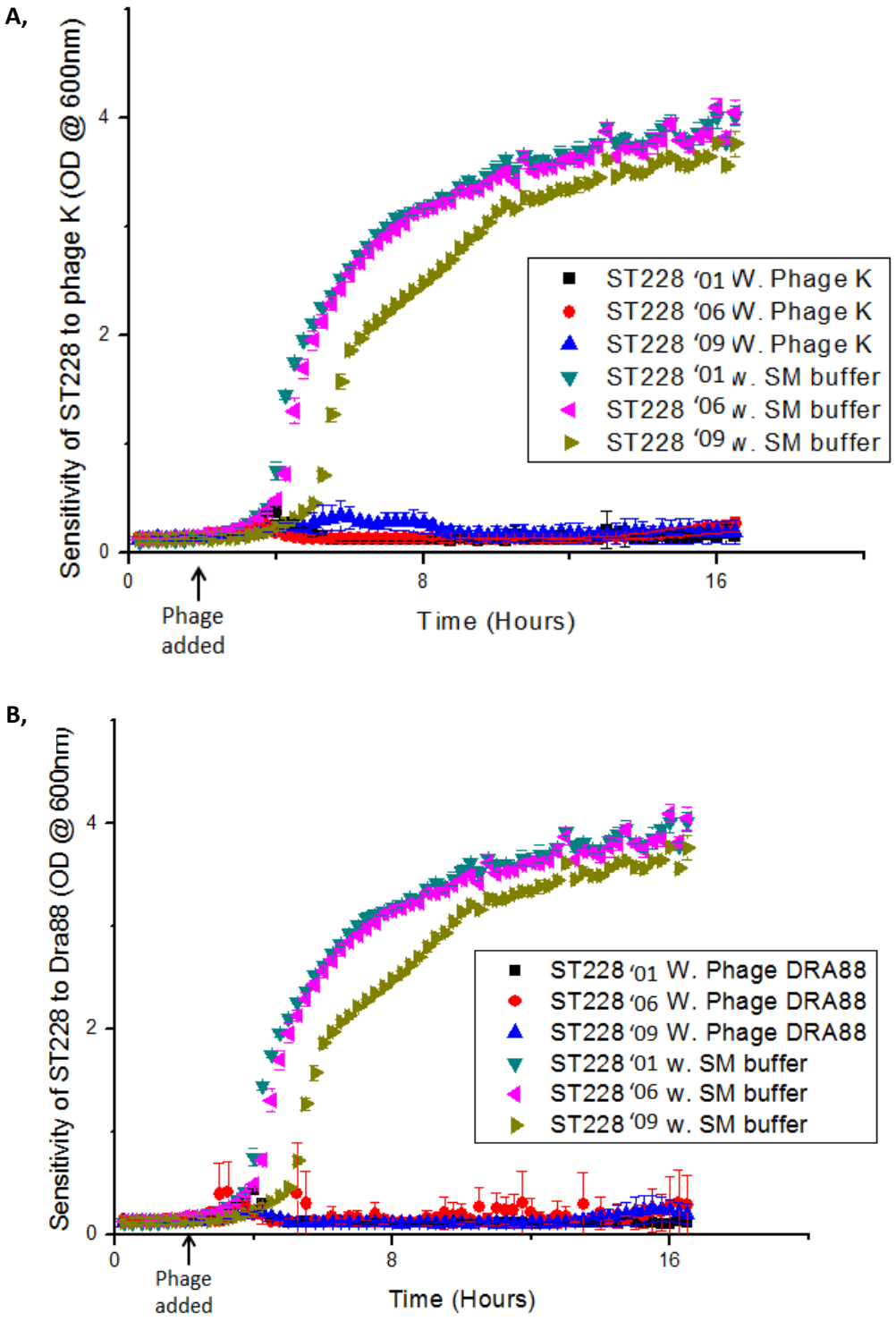

Figure 11 - The growth of ST228, as measured through OD600, isolated during 2001, 2006 and 2009, treated with Phage K (A) and Dra88 (B) in SM buffer (n=3). Error bars represent SD

In Figure 11 it is observable that both phages effectively lysed all isolates of ST228 studied, with little variation observable between samples isolated in 2006 (a non-outbreak year) and 2001/2009 (years in which an outbreak occurred). However, the collective data from the phage susceptibility experiments suggest that no difference in phage susceptibility occurred that could be correlated to the various ST228 outbreaks in Lausanne.

CONCLUSIONS

For the four phenotypes studied, biofilm formation, rhamnolipid vesicle lysis and phage susceptibility, no observable significant differences were seen between the representative subset of the collection studied. This suggested that external factors outside the scope of this investigation may be responsible for the outbreak. These factors might have a molecular basis outside of the genome. For instance, around 250 regulatory RNAs (sRNAs) are putatively involved in the intricate coordination of staphylococcal genes. The mRNA targets of a large proportion of these sRNAs is largely unknown (Guillet, Hallier and Felden, 2013).

Another potential factor that led to the ST228 outbreak might be the efficacy of the infection control protocol followed at the time. This might be the result of a small number of HCWs not adhering to the measures, or perhaps the measures taken were not comprehensive enough. Contamination of bed linen, for example, is increasing seen as a clinical fomite that often are not adequately decontaminated between patients (Creamer and Humphreys, 2008).

Finally, it is worth noting that the range of phenotypes tested in this study was far from comprehensive. Amongst the various other phenotypic factors that could have been included in this study were proteases, for instance staphopain B (a protease which degrades host fibronectin and fibrinogen) is putatively involved in *S. aureus* dissemination (Kolar *et al.*, 2013), fibrinogen- and fibronectin-binding or antibiotic resistance.

CHAPTER 3: DETERMINING NOVEL CANDIDATE GENES ASSOCIATED WITH PROTEASE PRODUCTION AND BIOFILM FORMATION THROUGH A GENOME-WIDE ASSOCIATION STUDY OF A POPULATION OF ST239

INTRODUCTION

ST239 is an epidemic clone of MRSA, accounting for a significant proportion of MRSA infections worldwide (Boswihi, Udo and Al-Sweih, 2016). The clone's success has been attributed in part to the variability of the gene expression of their virulence factors. In a comprehensive study of the variability of adhesin and toxin production in a collection of ST239 isolates by this group, taken from a variety of different types of infections in four Turkish hospitals, it was observed that a high degree of variability existed between the toxicity of these strains. Using GWAS, a number of genetic loci were seen to be significantly associated to toxicity, including a number of novel loci that were not previous considered toxicity regulating loci. In addition it was observed that a switch in expression between toxicity and adhesion genes is vital in facilitating the opportunistic nature of *S. aureus* (M. Laabei *et al.*, 2014b).

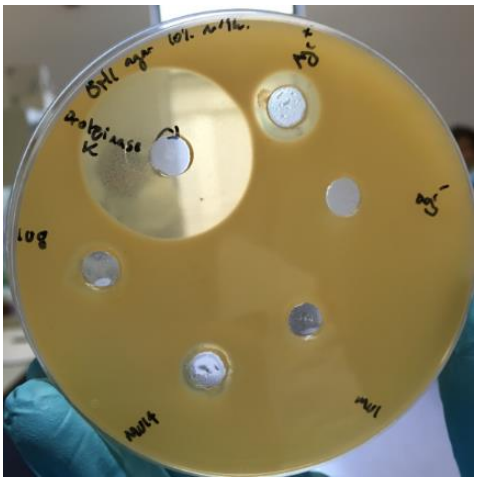

Figure 12 - 'Zones of clearance' observed on a standard milk agar plate

Given that both the whole genome sequencing data for the entire ST239 collection and the bioinformatics pipeline required to perform the GWAS were already in place, it was decided to use the approach of the study described about to test other phenotypes important to the virulence of ST239.

Protease production is a vital component of staphylococcal pathogenicity. A variety of proteases, which cleave different consensus amino acid sequences of their target proteins and are known to often auto-activate themselves in a proteolytic cascade that results in the zymogens of a number of major proteases (Aur, SspA, SspB and ScpA) activating to exert their extracellular function (Shaw *et al.*, 2004). However, little has been published regarding the regulation of these genes by pleiotropic loci, thus making protease activity an attractive

phenotype to study through GWAS. Another interesting phenotype that is important in MRSA virulence is biofilm formation. Biofilms are an integral means through which *S. aureus* persists on surfaces, defends itself further against antimicrobial action and concentrating environmental nutrients (Otto, 2008). Additionally, biofilms play a critical role in the progression of chronic disease; once a biofilm has been established in a host, individual cells can then disperse to colonise new sites of infection (Lister and Horswill, 2014).

The following chapter details the initial steps taken towards using GWAS to explore potential new genes important for protease production and biofilm formation within a collection of clinical MRSA-ST239 isolates.

RESULTS

Protease activity varies considerably between individual isolates of ST239

A subset of 95 isolates of ST239 samples were taken from four different university hospitals in Turkey between 2006 and 2009 from a variety of different sources, including wound infections, blood infections, catheters, spinal fluid and abscesses. Supernatant from the overnight cultures of these isolates were dispensed into wells within TSA with 10% milk and incubated overnight. The resultant 'zones of clearance' were formed from the supernatant diffusing out of the well, and any proteases within the supernatant degrading casein, the opaque protein found in milk, resulting in a clearance in the wells (Figure 14). The width of these zones of clearance, rounded to the nearest 0.25mm, was used as a high-throughput means of quantifying the overall protease production of each isolate. An *agr+* and *agr-* MRSA strain, RN6390B and RN4220 respectively, provided positive and negative controls, due to the established relationship between *agr* functionality and protease production (Lauderdale *et al.*, 2009) (RN4220 consistently and unambiguously produced halos of 1.5mm in diameter) while TW20 , the reference strain, was also included as a point of reference (Figure 13).

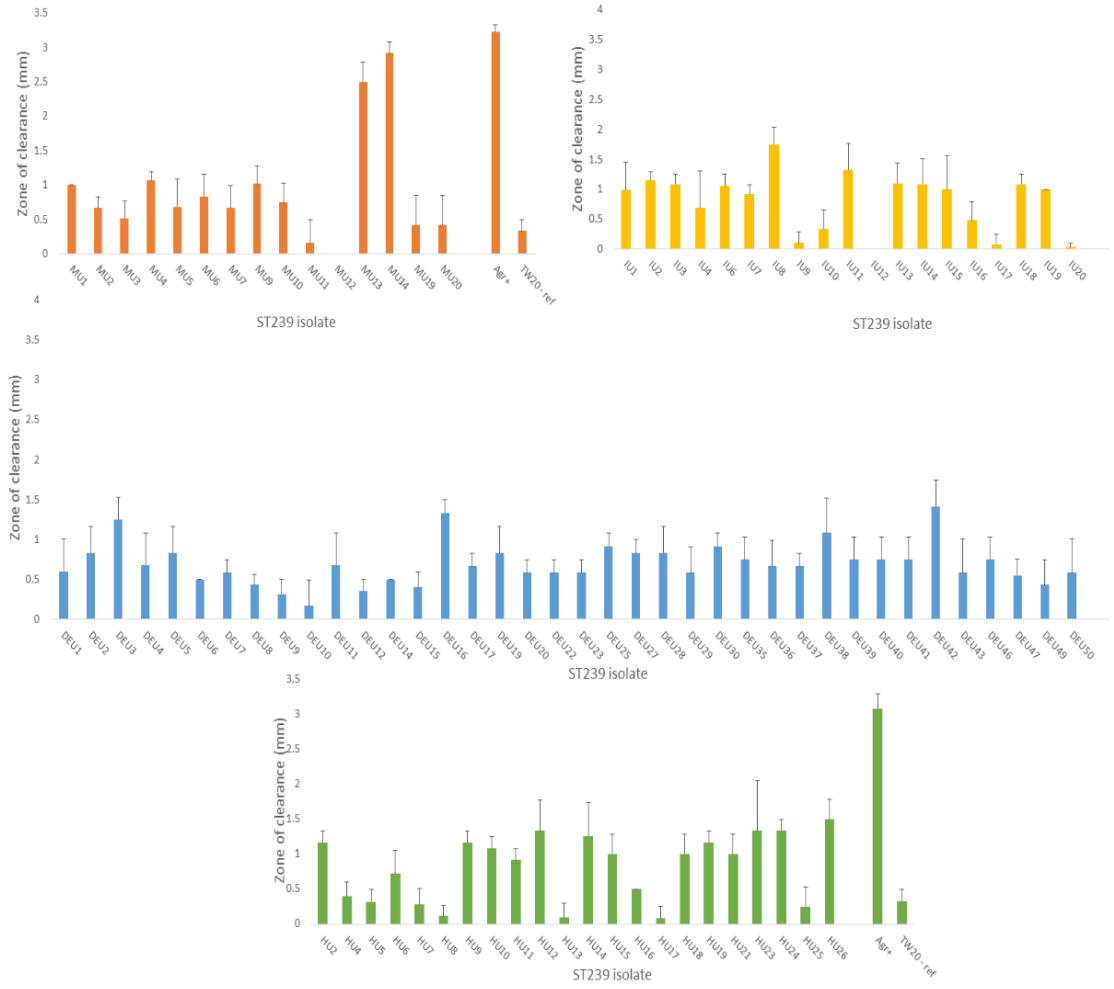

Figure 13 - Average protease activity of ST239 isolates taken from four hospitals in Turkey (MU, IU, DEU and HU taken from Marmara, Istanbul, Dokuz Eylul and Hacettepe University Hospitals respectively) measured through casein hydrolysis in milk agar plates (n=3). Error bars represent SD

The zones of clearance observed from the diffusion of the ST239 isolates supernatants through the milk agar showed some degree of variability (Figure 13), notably the diffusion of isolates MU12 and IU12 supernatants consistently did not result in any observable clearness in the agar surrounding the well whereas the supernatant of isolates MU13 and MU14 had zones of clearance comparable to the *agr+* control supernatants.

To assess whether any difference in the average protease production could be observed in samples isolated from different types of infections (abscesses, aspiration infections, bacteraemias, catheter related infections, spinal fluid infections, positive sputum samples,

positive tracheal secretion samples and positive wound swabs specifically), the results gained from the milk agar plate assay were analysed to account for the various sources of isolates (Figure 14).

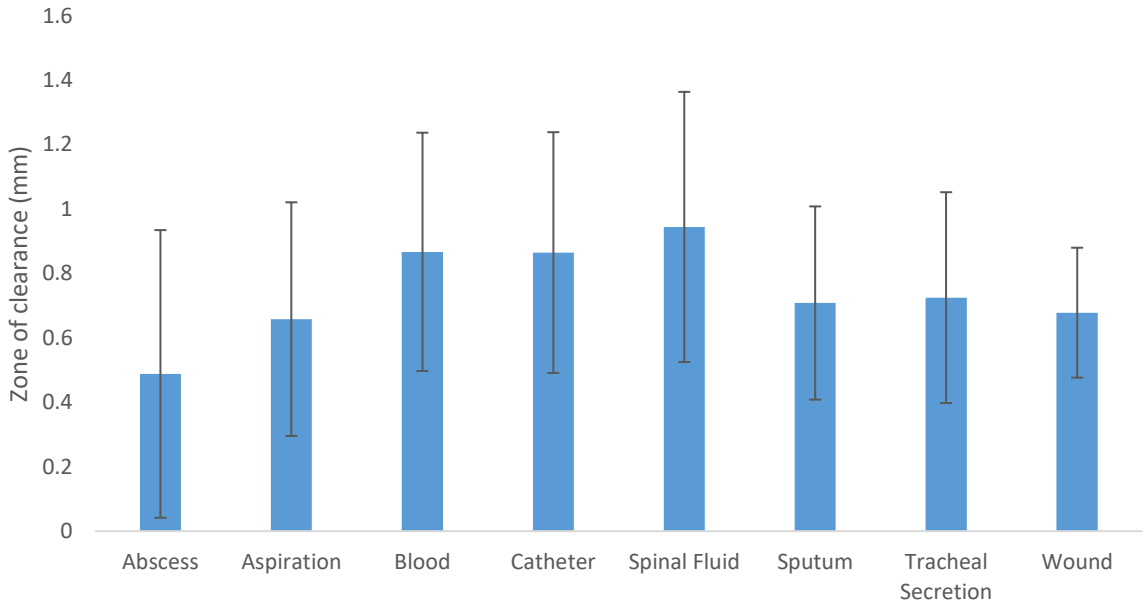

Figure 14 - Average protease activity of ST239 samples isolated from different types of infections of patients from four Turkish hospitals. Error bars represent SD

No significant differences were observed when the average protease production of samples isolated from abscesses, aspirations, blood, catheters, spinal fluid, sputum, tracheal secretion and wounds were calculated. Although this may be a consequence of having been grown in the same conditions, this suggests no intrinsic difference in protease gene expression under standard laboratory conditions.

SNPs with significant association to protease activity and transposon mutants in a USA300 FPR3757 background for verification of their significance

Using the protease data gained through the milk plate assay for the entire collection (apart from MU12, MU13 and MU14 due to lack of sequence data for these isolates) and previously collected whole genome sequencing data, significantly associated SNPs were found through data analysis using PLINK (carried out by Dr Mario Recker). Loci containing the top 50 SNPs were mapped against a USA300 FPR3757 reference genome and available transposon mutants within the Nebraska Transposon Mutant Library were located (Table 2).

Table 2 - Turkish ST239 SNPs with the highest P-value for protease activity when compared to the TW20 reference

P value for association	SNP location in TW20	Name/function of affected loci in TW20	Locus tag in FPR3757	Tn mutant in FPR3757
0.000187	2393426	*murA*	SAUSA300_2078	NE939
0.000187	2377659	*atpH*	SAUSA300_2061	NE1889
0.000187	2174068	*agrC*	SAUSA300_1991	NE873
0.000187	2997008	lip	SAUSA300_2903	NE338
0.000197	2775786	*sarU*	SAUSA300_2438	NE96
0.000272	2106193	hypothetical membrane protein	SAUSA300_1919	NE1805
0.000272	2845228	copper-translocating P-type ATPase	SAUSA300_2494	NE561
0.000272	1935622	oxidoreductase, aldo/keto reductase family	SAUSA300_1728	NE1441
0.000272	2178247	*scrR*, sucrose operon repressor	SAUSA300_1995	NE742
0.000272	1904373	cell wall surface anchor family protein	SAUSA300_1702	NE56
0.000272	2532617	putative membrane protein	SAUSA300_2211	NE627
0.000286	78396	*mecA*, penicillin-binding protein 2'	SAUSA300_0032	NE1868
0.000286	152937	NAD-dependent epimerase/dehydratase family protein	SAUSA300_0130	NE585
0.000286	967656	putative membrane protein	SAUSA300_0817	NE499
0.000312	1423837	catalase	SAUSA300_1232	NE1366
0.000349	435458	glycerol-3-phosphate transporter	SAUSA300_0337	NE1388
0.000349	1067541	*mgtE*, magnesium transporter	SAUSA300_0910	NE736
0.000349	1184963	*cyoE*, protoheme IX farnesyltransferase	SAUSA300_1016	NE1434
0.000349	214537	putative N-acetylmuramoyl-L-alanine amidase	SAUSA300_0181	NE1190
0.000349	1171002	*mntH*, manganese transport protein	SAUSA300_1005	NE1097
0.000349	1148420	hypothetical protein	SAUSA300_0982	NE1788
0.000349	15605	hypothetical protein	SAUSA300_0011	NE1827
0.000349	948986	PAP2 family protein	SAUSA300_0794	NE620
0.00043	78119	*mecA*, penicillin-binding protein 2'	SAUSA300_0032	NE1868
0.000453	1121452	*fmt*, fmt protein	SAUSA300_0959	NE1022
0.000656	1503504	*oppA*, oligopeptide ANC transporter, substrate-binding protein	SAUSA300_1300	NE440

0.000753	2147952	phi77 ORF014-like protein, phage anti-repressor protein	SAUSA300_0982	NE1786
0.000836	2753734	Intergenic region	Position 2601099	NE1482
0.000889	220518	*rocD*, ornithine aminotransferase	SAUSA300_0187	NE1069
0.000889	1029240	*cdr*, coenzyme A disulfide reductase	SAUSA300_0873	NE1456
0.000889	761112	Na+/H+ antiporter	SAUSA300_0617	NE1504

A variety of loci potentially affected by SNPs were determined, and isolates in a USA300_FPR3757 background with transposon insertions in regions homologous to these loci in the ST239 reference strain JE2 were identified. The rationale behind looking for these transposon mutants can be explained when the p values displayed in Table 2 are observed. GWAS are often prone to producing a high number of false positives due to linkage equilibrium (Power, Parkhill and de Oliveira, 2016). Thus, for example, the four 'most highly associated' loci (*murA*, *atpH, agrC* and *lip*) all have the same p value (0.000187), which suggests that only one of these loci are actually associated with the protease phenotype. As a result, validation of which loci are truly associated is required.

Functional verification of the effect of GWAS associated loci on protease activity

In an effort to elucidate which of the seemingly associated SNPs in certain genetic loci had a genuine effect on the average production of general proteases, transposon mutants in a USA300_FPR3656 background, with transposon insertions in genes corresponding to the affected loci in the ST239 cohort, were then isolated and the proteolytic activity of these mutants quantified through supernatant diffusion through milk agar (Figure 15).

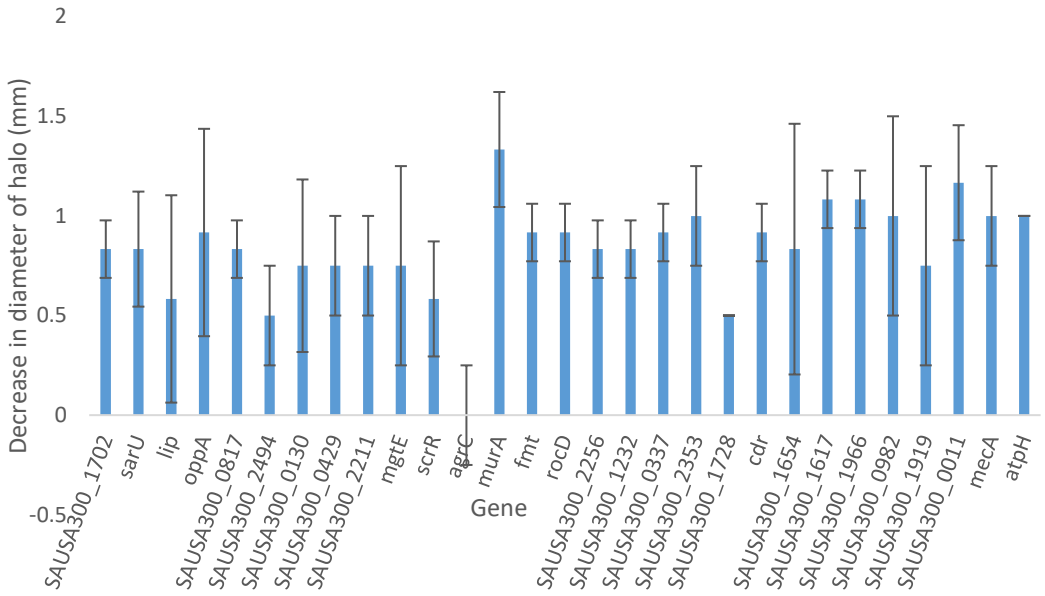

Figure 15 - The average decrease in protease activity of USA300_FPR3757 isolates with transposon insertions in the specified gene when compared to JE2. (n=3). Error bars represent SD

The top 50 SNPs that associated significantly with protease activity were used, which were relatively evenly distributed across the genome, with the exception of those that fell within the SaPI 1 (*S.* aureus Pathogenicity Island 1).

Every transposon insertion in genes affected by SNPs shown to have an association with a change in protease activity resulted in a decrease in protease activity, except, surprisingly, *agrC*, where inactivating the locus through the insertion of a transposon had a seemingly negligible effect on general protease production in USA300_FPR3757. Of the transposon mutants screened SAUSA300_2494 (a copper-translocating P-type ATPase encoding gene) and SAUSA300_1728 (an oxidoreductase, aldo/keto reductase family protein encoding gene) stood out as having the lowest decrease in protease activity when compared to JE2 (the USA300_FPR3757 reference strain), whilst *murA* had the largest decrease in protease activity. However, the USA300_FPR3757 background had significantly lower protease activity than the ST239 population, and the large degree of error seen in these data may be a consequence of the milk plate assays lack of sensitivity. Thus, meaningful conclusions from this dataset cannot be drawn.

An inverse correlation exists between general protease production and lytic activity in ST239

A previous analysis of lytic potential of these ST239 isolates was carried out and, interestingly, the isolate with the highest lytic potential HU13 had the sixth lowest proteolytic potential out of a cohort of 95 in the milk plate assay while the isolate MU9, with the lowest lytic potential, exhibited a moderate proteolytic phenotype. This prompted a brief comparison of average protease activity of the most and least toxic isolates of the Turkish ST239 collection, using the aforementioned vesicle lysis assay (data provided by Maisem Laabei) and the aforementioned measurements of casein hydrolysis by proteases present in bacterial supernatants in milk agar (Figure 16).

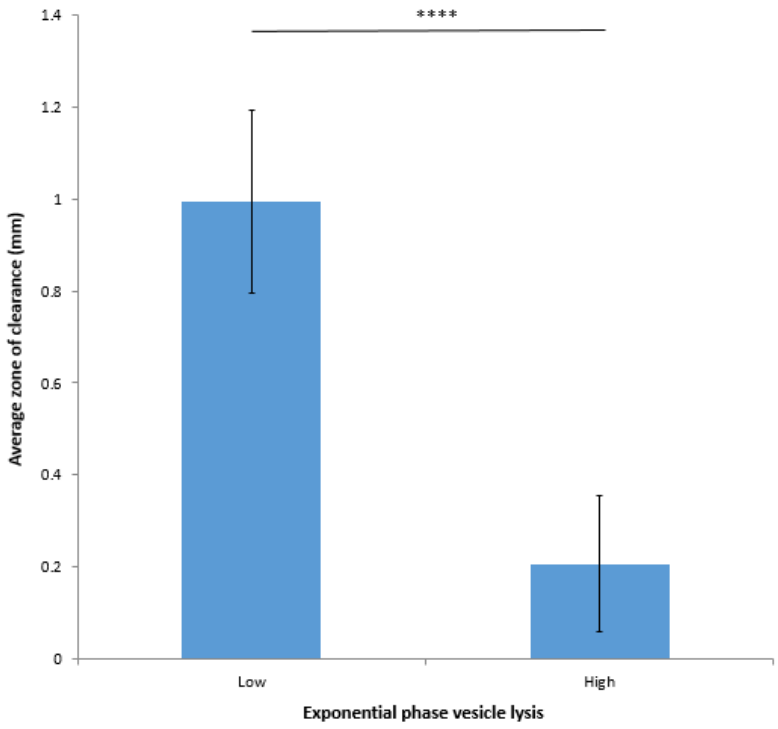

Figure 16 – The average protease activity of the most and least lytic ST239 isolates in the Turkish collection (n-10) (unpaired, single tailed Student's t test - p>0.0001 (denoted by ****, t=6.2343, df=18) *lytic activity determined through alpha toxin and PSM*

The average width of agar clearance in the ten ST239 isolates with the lowest lytic potential at the exponential phase of growth was significantly larger than that of the ten most toxic isolates (Figure 16). Interestingly, when this same comparison was carried out with the most and least lytic isolates of a collection of USA300 isolates (Maisem Laabei *et al.*, 2015) no significant different in proteolytic activity was observed.

Biofilm formation in ST239 isolates

Individual overnight cultures of the Turkish ST239 isolates were added in 1 in 40 dilution to TSB with 0.5% glucose, and the resultant biofilm formed at the bottom of each 96-well after incubation was stained with 1% crystal violet, the excess stain washed with PBS and the stained biofilm resuspended in 7% acetic acid. Through this the OD at 595nm can be used to quantify the biomass that accumulated at the bottom of the well. LAC and EMRSA-15 were both used as positive controls for this assay, which are done in triplicate on three separate 96 well plates, with three technical repeats per plates used. Variations per plate were then accounted for by blanking against the average OD of the TSB negative control wells and the results were shown as a percentage of EMRSA-15, a reliable biofilm former within ST239 (Smith *et al.*, 2008).

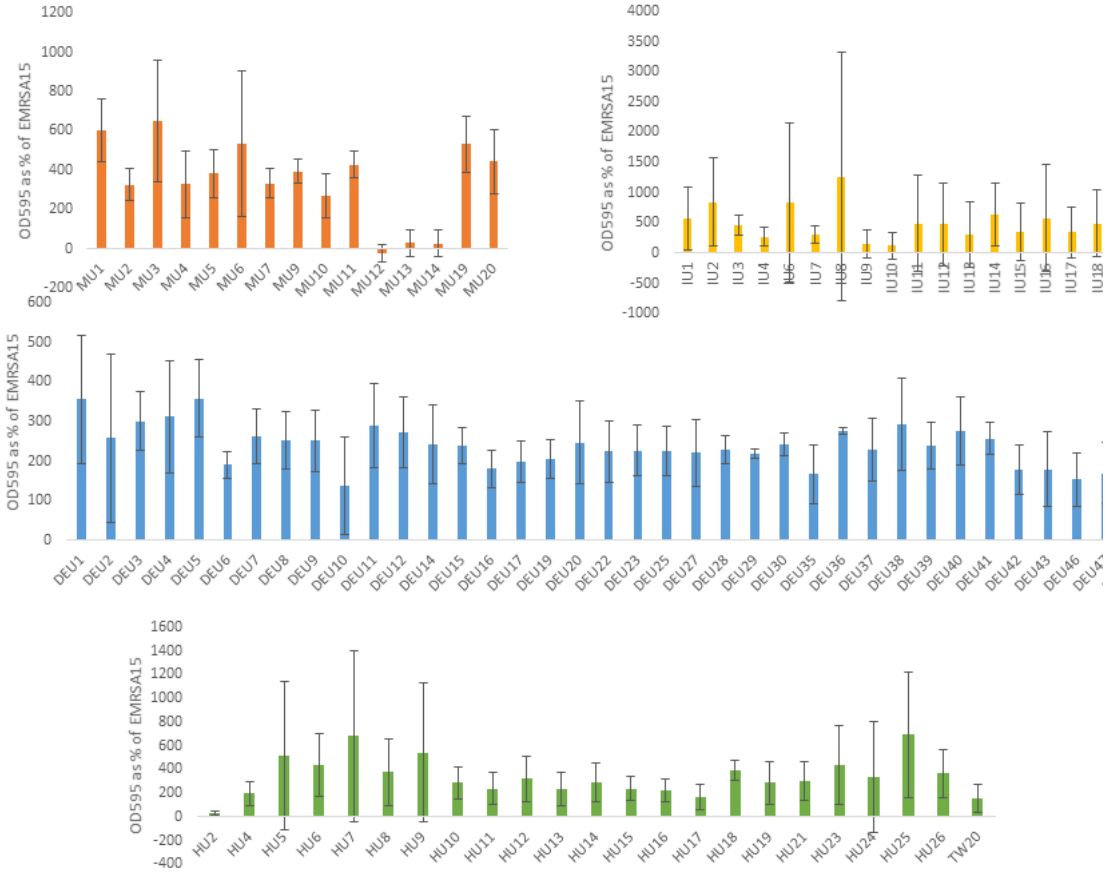

Figure 17 - Average biofilm formation of ST239 isolates taken from four hospitals in Turkey (MU, IU, DEU and HU indicting isolates taken from Marmara, Istanbul, Dokuz Eylul and Hacettepe University Hospitals respectively) measured through crystal violet staining (n=3). Error bars represent SD

As with the protease production data, despite the clonal nature of the collection, there was a large degree of variation observable within the collection as a whole, with some variation observable when the total average biofilm formation for individual hospitals are compared. Notably, MU12, MU13 and MU14 consistently failed to produce strong biofilms, as did IU9, IU10 and HU2.

SNPs with significant association to biofilm formation and transposon mutants in a USA300 FPR3757 background for verification of their significance

Using the biofilm data gained through the crystal violet staining assay for the entire collection (apart from MU12, MU13 and MU14 due to lack of sequence data for these isolates) and previously collected whole genome sequencing data, significantly associated SNPs were found through data analysis using PLINK (carried out by Dr Mario Recker). Loci containing the top 50 SNPs were mapped against a USA300 FPR3757 reference genome and available transposon mutants with the Nebraska Transposon Mutant Library were located, the most relevant of which are listed in Table 3.

Table 3 - Turkish ST239 SNPs with the highest -log P-value for biofilm formation when compared to the TW20 reference

-log of P value for association	SNP location in TW20	Name/function of affected gene in TW20	Locus tag in FPR3757	Tn mutant in FPR3757
1698548	1684374	hypothetical protein	SAUSA300_1534	NE1210
17463	17392	ccrB (cassette chromosome recombinase B)	SAUSA300_0037	NE495
655782	584731	methyltransferase small subunit	SAUSA300_0526	NE1671
1982993	1958590	hypothetical protein	SAUSA300_1771	NE68
2514045	2362155	*secY*	SAUSA300_2184	No Tn
2637384	2485589	*mqo* (malate:quinone oxidoreductase)	SAUSA300_2312	NE1003
2987716	2821611	hypothetical protein	SAUSA300_2593	NE1618
366127	363875	*nupC* (putative nucleoside permease)	SAUSA300_0313	NE622
428285	384011	*sgaT* (transport protein)	SAUSA300_0330	NE758
553689	482991	*mecA* (PAP2 family protein)	SAUSA300_0429	NE620
565190	496216	hypothetical protein	SAUSA300_0443	NE1831
1545621	1488076	cell surface protein	SAUSA300_1327	NE1
1641720	1614525	6-phosphogluconate dehydrogenase	SAUSA300_1459	No Tn
1671901	1657762	hypothetical protein	SAUSA300_1505	NE377
2520835	2368497	*rpsS* (30S ribosomal protein S19)	SAUSA300_2200	No Tn
2603650	2451267	*hutU* (urocanate hydratase)	SAUSA300_2278	NE435
2681927	2529787	Zn-binding lipoprotein adcA-like protein	SAUSA300_2351	NE568
2731802	2578578	an amino acid permease	SAUSA300_2395	NE1131
211613	203715	non-ribosomal peptide synthetase	SAUSA300_0181	NE119
830266	758921	fructose specific permease	SAUSA300_0685	NE768
1834421	1814651	*dnaE* (DNA polymerase III alpha subunit)	SAUSA300_1649	No Tn

A variety of loci potentially affected by SNPs were determined, and isolates in a USA300_FPR3757 background with transposon insertions in regions homologous to these loci in the ST239 reference strain JE2 were identified. Again, due to the prevalence of false positives in GWASs, validation of which of the listed loci in Table 3 have a true association with biofilm is necessary before any conclusions can be drawn.

Functional verification of the effect of GWAS associated loci on biofilm formation

In the same vein as the approach taken in the study of protease activity for this collection of isolates, to try and eliminate false positive associations, transposon mutants in the USA300_FPR3656 background, with transposon insertions in a number of the candidate genes listed in Table 3 were isolated and biofilm formation was quantified through microtiter crystal-violet staining (Figure 19).

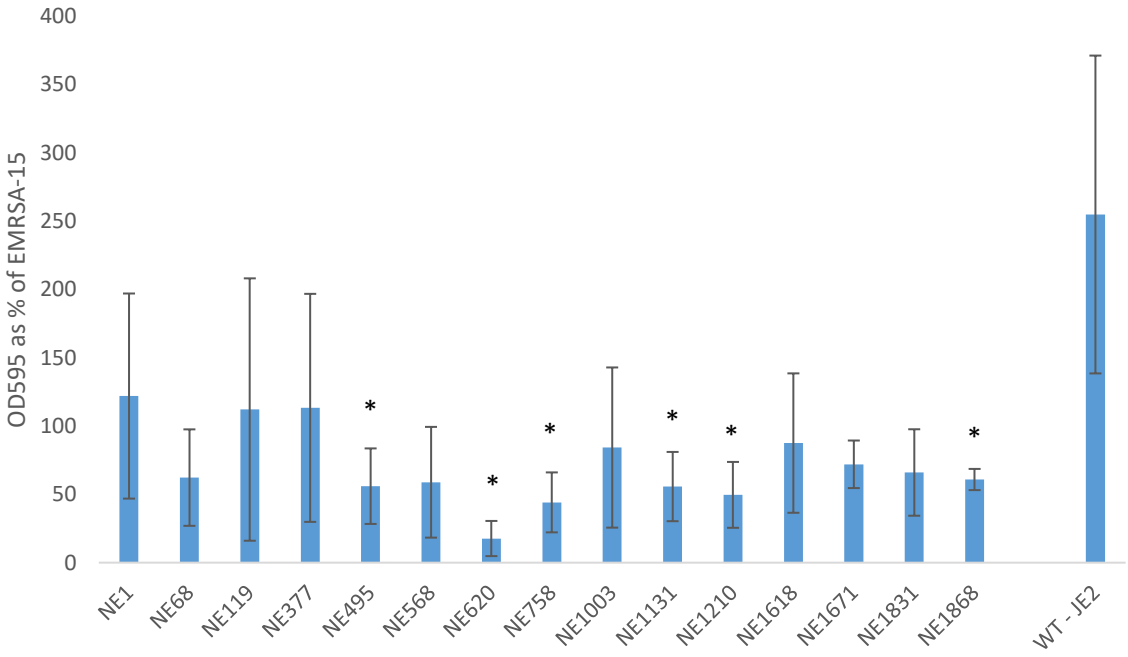

*Figure 18 - The average biofilm formation of USA300_FPR3757 isolates with transposon insertions in GWAS generated candidate loci (see Table 3 - Turkish ST239 SNPs with the highest -log P-value for biofilm formation when compared to the TW20 reference, compared to JE2 background strain (n=3). Error bars represent SD. * denotes p<0.05 between a transposon mutant and the JE2 wild type*

The average biofilm formation of candidate genes generated from the GWAS study of the ST239 cohort were studied using analogous transposon mutants in the USA300_FPR3757 background whose ability to produce biofilms were compared to that of JE2, the reference strain for this collection. A two-tailed, paired T-test showed the following transposon mutants average biofilm formation were significantly lower ($p < 0.05$) than the JE2 reference strain (P values are as follows: NE495, p=0.00449; NE620, p=0.024669; NE758, p=0.0368; NE1131, p=0.0442; NE1210, p=0.0402; NE1868, p=0.0449). These numbers represent isolates from the USA300_FPR3757 with transposon mutants in the following genes respectively; *ccrB*, *mecA*, *sgaT*, a gene responsible for encoding an amino acid permease and a hypothetical protein (for convenience, these shall be referred to as *aap* and *hyp* respectively for the remainder of this report). These genes were therefore chosen for further analysis for their effect on the biofilm formation capabilities of USA300_FPR3757 through complementation analysis.

Complementation of candidate genes involved in biofilm formation

Following the establishment of a list of candidate genes with a potential association with biofilm formation (Table 3) and the comparison of the biofilm production of these transposon knockouts against the USA300_FPR3757 reference strain, 5 candidate genes (*mecA*, *ccrB*, *sgaT*, *aap*, and *hyp*) were amplified in the purified genome from wild-type JE2, through the use of primers containing appropriate restriction sites flanking the insert (see Appendix 1). These amplicons were then cut using the appropriate restriction enzymes and ligated between the corresponding cut restriction sites within the tetracycline inducible plasmid pRMC2. The success of the ligation of the amplicon to the pRMC2 backbone was assessed through 1% agarose gel electrophoresis.

Successfully spliced vectors were transformed into chemically competent *Escherichia coli* vectors, DH5α, through heat shock. These transformed DH5α isolates were then propagated in media containing tetracycline to select for successful transformants. pRMC2 plasmids containing one of the 5 candidate genes was purified, amplified and then inserted into a second conduit, RN4220, through electroporation to ensure that the resulting post-translational modifications on the plasmids are appropriate for insertion into their corresponding transposon mutants (reducing the possibility of the insert being degraded). Transformed RN4220 propagation and DNA purification and amplification occurred as previously described, with the resultant plasmids (each containing one of the five

aforementioned candidate genes) were then electroporated into the corresponding transposon mutant. A crystal violet staining assay was then used to compare the biofilm formation of the transposon mutants to their complemented counterparts (Figure 19).

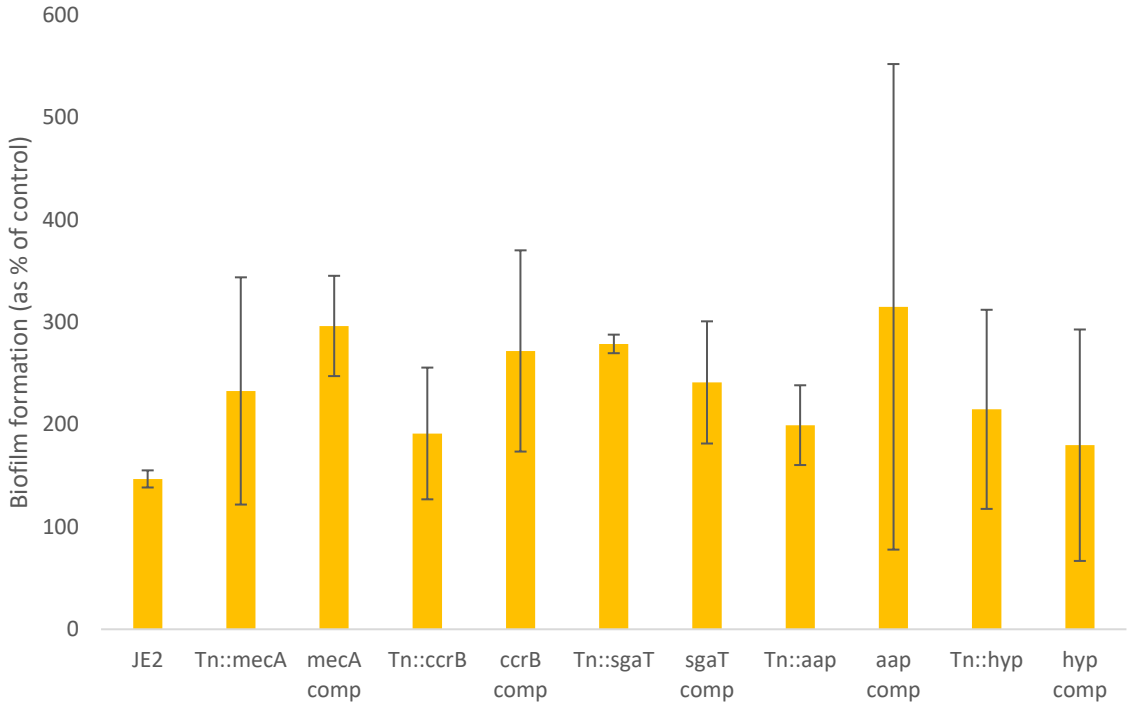

Figure 19 - Biofilm formation (as a percentage of OD595 of MRSA-15) of transposon mutants of candidate genes and their compliments (n=9). Error bars represent SD

For the 5 candidate genes focused on here (*mecA*, *ccrB*, *sgaT*, *aap* and *hyp* respectively), no significant difference in biofilm formation was observed between the transposon mutants and isolates that had a functional version of the candidate gene reintroduced (Figure 19)(paired student t-test p values as follows: *mecA* (p=0.137), *ccrB* (p=0.118), *sgaT* (p=0.183), *aap* (p=0.269) and *hyp* (p=0.339)). The biofilm formation for the majority of the transposon mutants also now had, curiously, no statistical difference in biofilm formation against the wild type reference JE2, calling the integrity of the dataset into question.

Correlations between the all currently known phenotypes of the Turkish ST239 collection

Previous work carried out by this lab have investigated a number of other phenotypes of these 95 ST239 isolates, including fibrinogen binding, fibronectin binding and lytic activity (due to δ toxin, PSM-alpha1, -alpha2 and –alpha3 production). With the addition of biofilm

formation and protease production added to this wealth of phenotypic information, a series of Pearson's rank correlation tests were performed on the data set to try and elucidate whether any observable correlations existed between all the phenotypes tested (Table 4).

The only phenotype that showed a significant correlation to protease activity was vesicle lysis at the exponential stage of culture growth (Table 4) as mentioned previously. All positive correlation between fibrinogen and fibronectin binding is as expected, however, the lack of other statistically significant correlations between the other phenotypes described is worthy of note.

Table 4 The Pearsons' rank correlations of various phenotypes known for a collection of ST239 isolates taken from four Turkish hospitals. Acronyms represent the following phenotypes (Fb; Fibrinogen binding, Fn; Fibronectin binding, VL; Vesicle lysis (measuring δ toxin and PSMs production, Pr; Protease production, Bio; Biofilm formation) (-Ex/-St denote the phase of growth of the isolates at which measurements were taken: exponential phase and stationary phase respectively). Figures represent the raw Pearsons' rank correlation coefficient data, with figures highlighted in green representing statistically significant linear positive correlations between two phenotypes, while those in blue highlight statistically significant linear negative correlations between two phenotypes (p>0.05)

Pearsons rank Correlation coefficients	**Fb-Ex**	**Fb-St**	**Fn-Ex**	**Fn-St**	**VL-Ex**	**VL-St**	**Pro-Ex**	**Bio-Ex**
Fb-Ex		0.526	0.61	0.552	-0.051	-0.076	0.152	-0.079
Fb-St	0.526		0.496	0.842	0.029	-0.147	0.204	-0.146
Fn-Ex	0.61	0.496		0.638	-0.292	-0.201	0.166	-0.275
Fn-St	0.552	0.842	0.638		-0.12	-0.106	0.169	-0.133
VL-Ex	-0.051	0.029	-0.292	-0.12		0.284	-0.226	0.288
VL-St	-0.076	-0.147	-0.201	-0.106	0.284		-0.622	-0.022
Pro-Ex	0.152	0.204	0.166	0.169	-0.226	-0.622		0.042
Bio-Ex	-0.079	-0.146	-0.275	-0.133	0.288	-0.022	0.042	

Discussion and Conclusions

General protease production was assayed through a high-throughput decanting supernatant into milk agar plates. Clear differences were observed within the cohort of these highly related clones, MU13 and MU14 both exhibiting zones of clearance around 5x larger in width than the average for isolates from that hospital, while MU12 and IU12 supernatants consistently did not produce any observable clearance. Despite the high degree of variation of protease production in isolates from within the same hospital, little variation was observable between isolates from the different hospitals. When transposon mutants in a USA300_FPR3757 background with transposon insertions in homologous regions to the various potentially associated loci were analysed for protease production using the milk plate assay were analysed, however, no clear differences in the transposon mutants protease production was observed. This is the likely consequence of the USA300_FPR3757 background not being as proteolytic as ST239, suggesting that a more sensitive assay is required for more consistent results. When protease activity was compared to toxicity in the ST239 collection, an inverse relationship was observed, however, given the multiple regulators that may be influencing both phenotypes, the exact regulators responsible for this trend have not been determined.

Within the cohort of ST239 isolates, a high degree of variation in biofilm formation was observed overall in the population, making biofilm formation another interesting candidate phenotype to study further through GWAS. An interesting observation when the entire cohort's phenotypic data were statistically compared was the lack of correlation between biofilm formation and toxicity, as both phenotypes are tightly regulated by the *agr* quorum sensing system. Within the *agr* system, δ toxin (one of the toxins the aforementioned vesicle lysis test is sensitive to) is encoded within the coding sequence of RNAIII: the *agr* systems effector molecule. Most PSMs are upregulated in the presence of agrA (Queck *et al.*, 2008). The *agr* system has also been described as an important factor in the initial stages of biofilm formation (Boles and Horswill, 2008). However, this may be a consequence of the large standard deviations produced by the assay (Fig. 21) which is an expected outcome of a microtiter biofilm assay with particularly strong biofilm formers. This is due the assays propensity to detach more loosely adhered biofilms during wash steps, particularly at a higher biomass (Joana Azeredo *et al.*, 2017b).

Five candidate genes (*mecA*, *ccrB*, *sgaT*, *aap* and *hyp*) were shown to have a potential association with biofilm formation, however subsequent complementation analysis failed to verify this conclusively.

DISCUSSION

AIM 1: SURVEY CHANGES TO THE MICROBIAL BURDEN OF A BURN'S UNIT CAUSED BY ITS RE-LOCATION

The relocation of the Bristol Children's Burns Unit into a larger, more modern hospital offered a rare opportunity to study any changes in the ecology of hospital-pathogens within the niche clinical environment of a paediatric burns unit. A number of swabs were taken from surfaces and fomites within several rooms in the ward, as well as swabs of the hands, nasal passages and identification cards of consenting healthcare workers that worked at the unit. These swabs were taken both before the relocation and after, with an additional set of swabs taken in the newly emptied and cleaned new ward prior to the move.

However, unfortunately, all samples collected as part of this sub-project were propagated in TSB at 37^{o}C. In hindsight, this error hampered the project, resulting in the samples obtained no longer representing the true microbial diversity of the clinic. This demonstrates the value of thorough experimental design, with particular attention given to the sampling – the most important step in any metagenomics project (Thomas, Gilbert and Meyer, 2012b).

General advice regarding isolation and extraction require a large yield of high quality nucleic acids that are representative of the diversity of the microbial community sampled. Commercially available DNA isolation kits designed for metagenomic analysis are often designed for testing soil or water sample contamination. This is because propagating bacterial populations using enriched nutrient media, such as TSB (chosen in this instance for its ability to support the growth of a wide-variety of microorganisms) will invariably influence the population dynamics of a sample (Greninger *et al.*, 2017).

A more appropriate approach to the sample collation may have been through directly inoculating water with the swabs used to take the samples as opposed to using the charcoal Amies transport medium used in this instance. Additionally, a prudent measure during the early stages of this investigation would have been to have developed a tested, bespoke protocol for the isolation of metagenomic DNA from environmental swabs prior to the hospitals relocation. This would have also informed the approach planned for the subsequent stages of the study. For example, given that certain types of samples (such as ground-water or

biopsies) have been shown to provide DNA yields too low for sequencing, amplification of the starting genetic material prior to sequencing may well have been required post-sampling, most commonly through multiple displacement amplification (Thomas, Gilbert and Meyer, 2012b).

Should the microbial diversity not have been skewed through sampling error, the intended next step would have been to using shotgun metagenomic sequencing, an approach that randomly shears all DNA in a sample into fragments which can then be sequencing through standard next-generation sequencing methods. If sequenced deeply enough, the resulting reads were then intended to be assembled using a metagenomic analysis framework capable of profiling at the subspecies level, PathoScope 2.0 (Hong *et al.*, 2014). The output from this analysis would have provided important information regarding the ecology of both benign and pathogenic bacteria within a clinical setting and, crucially, provided information that could then have been used to observe any shift in transmission dynamics caused by the relocation. Another interesting avenue to explore might have been assessing which colonisers of the ward environment played the largest role in the recolonization of the ward environment.

AIM 2: IDENTIFY PHENOTYPIC DIFFERENCES ASSOCIATED WITH A *S. AUREUS* OUTBREAK IN A TERTIARY CARE HOSPITAL.

The consecutive ST228 outbreaks seen in a Swiss tertiary care hospital (Figure 6) over a ten-year period represented an opportunity to explore whether phenotypic variations can be used to account for outbreaks, after phylogenetic analysis of eight isolates from various years showed that the epidemic clones were close relatives, leading to the conclusion that the genomic data alone was not sufficient in explaining the markedly increased incidence of ST228 in 2008. In an attempt to gain some understanding of the molecular mechanisms that might have been responsible for the repeated resurgence of this clone, a “phenotype-first” approach to exploring epidemics might give some indication of what drove this clones rapid dissemination.

Biofilm formation, δ toxin and PSM production and phage susceptibility were studied in ST228 isolates taken throughout the 10-year period of study. Biofilm formation was studied

quantitatively (OD_{595}) through crystal-violet staining, the most common method of investigating biofilm formation in bacteria (J. Azeredo *et al.*, 2017a). Biofilms play an important role in transmission and survival of MRSA, both factors which are essential to epidemic behaviour (Vanhommerig *et al.*, 2014). The formation of biofilm matrices afford the community of bacteria within added protection against external stressors (including antimicrobials), allowing them to persist longer in clinical environments (Lister and Horswill, 2014), making biofilm formation a phenotype that might have contributed to the Lausanne outbreaks. However, the data presented in Figure 8, representing the production of biofilms by ST228 isolates taken during the surveillance programme established at the University Hospital of Lausanne between 2000 and 2009, showed abnormally low biofilm production in all isolates tested. While microtiter plate dye-staining approaches to measure biofilms have the advantage of being high-throughput and versatile, a well-known limitation of this technique is the experiments lack of reproducibility, accounting for the large degree of error. Another limitation of this technique is the frequency with wish biofilm biomass is overestimated or underestimated, depending on the washing step (J. Azeredo *et al.*, 2017a). This is a likely explanation for the unexpectedly low biofilm biomass seen in Figure 8. The dataset is also made poorer by the lack of positive control used during the data collection, which may have been used in order to standardise the dataset to account for potential over-washing of the biofilm.

PSMs are a family of secreted peptides with multiple functions in staphylococcal pathogenesis. PSMs exert their influence over staphylococcal pathogenicity through the recruitment and lysis of neutrophils. There are seven PSM genes encoded for by the *S. aureus* core genome, including δ-toxin (Kretschmer *et al.*), and are putatively regarded as more frequently produced in more pathogenic strains of *S. aureus* (Cheung *et al.*, 2014). The operons *psmα, psmβ* and RNAIII (encoding δ-toxin) are all under the strict control of the the AgrA DNA binding protein, with PSMs playing a vital role in biofilm detachment and strain dissemination (Periasamy *et al.*, 2012). As a result, production of PSMs was another phenotype investigated in order to attempt to gain insights into the molecular processes that governed the Lausanne outbreaks. Figure 9 demonstrates, through the use of a lipid vesicle lysis assay, that ST228 isolates from both outbreak years and non-outbreak years displayed low PSM production, remaining consistent with the avirulent control, DH5α. This coincides with previous work categorising ST228 as a strain that produces low amounts of PSMs

(Rasigade *et al.*, 2013). No difference in PSM production was observed in isolates taken from outbreak period when compared against those isolated outside of the epidemics. Thus it came be inferred that a change in PSM production is unlikely to be a factor that influenced the ST228 outbreaks.

ST228 isolates taken from outbreak and non-outbreak periods were also tested for their susceptibility to lysis by bacteriophage (Figure 11). Phage are now understood to play an important role in determining the microbial ecology of an environment, as well as acutely influencing microbial evolution (Donlan). Lytic phage complete their life cycle through invading and propagating within a bacterial host, mediating competition among strains in the process (Meaden and Koskella, 2013). With this in mind, we explored whether there was a relationship between the susceptibility of ST228 isolates to two available phage, Dra88 and phage K, and the year of isolation. However, no difference in susceptibility to phage lysis was observed in any of the isolates examined (Figure 11). This suggests that the elevated incidence of cases of infection by ST228 in 2008 was not the result of an evolutionary change in phage susceptibility. If this were the case, it would be reasonable to expect the ST228 isolate from 2009 to grow significantly more in the presence of bacteriophage than the other isolates tested. That said, however, no concrete inferences can be made from these data. This may be a consequence of either an insufficient number of biological repeat for each year carried out or due to the genetic similarity of the two phage used (Alves *et al.*, 2014).

AIM 3: DETERMINE NOVEL CANDIDATE GENES ASSOCIATED WITH PROTEASE PRODUCTION AND BIOFILM FORMATION THROUGH A GENOME-WIDE ASSOCIATION STUDY OF A POPULATION OF ST239

ST239 Protease Screening

S. aureus virulence depends significantly upon the expression of a secretese of proteases, including cysteine proteases, staphopains A and B (ScpA and SspB respectively), zinc-dependent metalloprotease aureolysin (Aur) and several serine proteases (Zdzalik *et al.*, 2012). These proteases are positively regulated by the action of *agr* and negatively regulated by *sarA* and play numerous roles, including nutrient acquisition, evasion of host immunity by interacting with plasma proteins and antimicrobial peptides (Pietrocola *et al.*, 2017). A screen of a population of MRSA-ST239 isolates from four tertiary care hospitals in Turkey using

casein agar showed that protease production varied throughout the collection (Figure 13), however, when these data were grouping into the types of infections the samples originated from, no difference in average protease production was observed (Figure 14). A number of loci with a potential association with protease production were found to be within mobile genetic elements known as SaPIs, superantigen pathogenicity islands. The SaPIs are mobile phage-related pathogenicity islands that often harbour at least one superantigen gene (Alibayov *et al.*, 2014). The high association between SNPs in genes within SaPIs and changes in protease activity may be expected, since it has been documented that the expression of superantigens, particularly TSST-1, pleiotropically represses the expression of the major protease gene *sspA* (V8 protease) (Calander *et al.*, 2008). While the study of these loci was omitted from this study in the interest of time, future analysis of these loci and their relationship to proteolysis in *S. aureus* may well be warranted.

Select transposon mutants with transposon insertions in genes affected by SNPs shown to have a potential association with a change in protease activity resulted in a decrease in protease activity, except *agrC*, where inactivating the locus through the insertion of a transposon had a negligible effect on general protease production in USA300_FPR3757. SAUSA300_2494 (a copper-translocating P-type ATPase encoding gene) and SAUSA300_1728 (an oxidoreductase, aldo/keto reductase family protein encoding gene) had the smallest decrease in protease activity when compared to JE2 (the USA300_FPR3757 reference strain), whilst murA had the largest decrease in protease activity (Figure 14).

The *agr* system facilitates the detachment of biofilms through its interference with protease expression. AgrC is a membrane protein that binds to autoinducing peptide (AIP), a quorum sensing molecule secreted during growth and responsible for activating gene expression in a cell density dependent manner (Olson *et al.*, 2014). Increasing levels of exogenous AIP, detected through AgrC, functions as a biofilm detachment signal. This activates the *agr* system and leads to the upregulation of extracellular proteases, which work to degrade the biofilm and signals a switch into a planktonic state (Boles and Horswill, 2008). As a result, the interruption of the *agrC* gene by the transposon insertion having a negligible effect on protease production is surprising.

This departure from the anticipated effect of an *agr* knockout may call into question the reliability of the data collected. The method used to assess protease production involved the

manual measuring of the wide of the zone of clearance, made through casein cleavage by common extracellular proteases had a clear limitation – measurements were made by eye with a ruler in order to provide a quantitative measurement for protease activity. While every effort was made to ensure the consistency and accuracy of the measurements, a more robust assay to screen protease production may have resulted in a clearer dataset.

That said, however, the fact that the protease data for the ST239 cohort shows a strong negative correlation against δ -toxin production (Figure 16) might actually validate that the milk plate assay was sufficient in measuring protease activity. Many master regulators (including the *agr* operon and the *sarA* system) regulate both protease activity and toxin production (Bronner, Monteil and Prévost, 2004). However, the exact regulators involved are not clear. For example, the *agr* system is well known as an important upregulator of toxins (δ toxin itself for instance, one of the toxins assayed through the vesicle lysis test, is a component of RNAIII, a direct transcriptional product of the *agr* operon), however it has also been shown that the *agr* system upregulates *aur*, *ssp* and *scp*, three important protease genes (Shaw *et al.*, 2004). That said however, sarA has been described as a potent downregulator of protease gene expression (Bronner, Monteil and Prévost, 2004) and, in the case of PSMs, the downregulation of the protease aureolysin in particular reduces post-secretion degradation of PSMs (Rasigade *et al.*, 2013), which may account for the negative correlation between the two phenotypes. Further comprehensive analysis of the potential regulators of ST239 is warranted to elucidate the true regulatory systems that govern this inverse correlation between protease production and toxicity.

During the project, an attempt was made at developing a microtiter plate assay, measuring proteolytic activity in a sample through optical density readings made after a colormetric test. The proposed assay, modified from (Cupp-Enyard, 2008), aimed to measure the degradation of casein through the reaction between liberated tyrosine and Folin and Ciocalteus Phenol reagent, a reaction which produces a blue-coloured chromophore (Cupp-Enyard, 2008), however, the optimization of this assay for bacterial supernatants was not completed during the project. That said, another potential reason for the lack of conclusive data regarding the protease production of the transposon mutants could be a difference between how proteolytic the USA300_ FPR3757 background is compared to ST239. Further testing, however, would be required to qualify this.

ST239 Biofilm Formation Screening

Biofilm formation is an important clinical phenotype: responsible for over 60% of microbial infections, and 80% of chronic infections (Gutiérrez *et al.*, 2017). Through a modified version of crystal violet staining assay with fewer wash steps, biofilm formation of the ST239 collection was quantified (Figure 19). Transposon knockouts for candidate loci with the potential of having an association with biofilm formation were isolated, and compliments for 5 of these candidate genes were produced.

These genes are as followed: *mecA* (carried within SCC*mec*) which encodes the penicillin-binding protein PBP2a (Wielders *et al.*, 2002)); *ccrB*, also carried on the SCC*mec* element, encodes a site-specific recombinase that putatively mediates both the integration and excision of SCC*mec* into/out of the staphylococcal core genome (Wang and Archer, 2010); *sgaT*, a transmembranous putative part of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS); *app*, an uncharacterized amino acid permease and *hyp*, an uncharacterized hypothetical protein.

The apparent association between the SCC*mec* element and biofilm formation is not a novel one, with significantly higher rates of biofilm development in strains with greater antibiotic resistance having been previously reported, dispelling the previous assumption that increased levels of antibiotic resistance in “biofilm-positive” MRSA can wholly be attributed to the biofilm acting as a physical barrier against the action of antibiotics (Kwon *et al.*, 2008; Reiter Keli *et al.*, 2011). In *ica*-independent biofilm formation (the *ica* locus having been shown to be redundant for MRSA biofilm formation (O'Neill *et al.*, 2007)), the action of PBP2a (produced by *mecA*, thus also involving *ccrB*) represses the *agr* system which, as a result, downregulates the production of PSMs, proteases and virulence determinants. PBP2a concurrently also upregulates biofilm-production, due to the putative down regulation of *agr*, thus inhibiting the *agr*-mediated upregulation of the proteases responsible for biofilm dispersal (McCarthy *et al.*, 2015).

Phosphotransferase system (PTS) transporters are the predominant class of bacterial carbohydrate transporters, which are responsible for importing exogenous sugars into the cell as well as modifying the sugars during their transport through the membrane. Glucose has been previously described as a substrate capable of influencing the expression of *S. aureus* virulence factors. Biofilm-related genes *cidA* and *icaA* are upregulated in the presence of

exogenous glucose, alongside the glucose-mediated downregulation of the *agr* system (Vitko *et al.*, 2016).

Mature *S. aureus* biofilms maintain a dynamic flux of metabolic activity, uptaking and excreting both carbon and amino acid regularly (Zhu *et al.*, 2007). Branched-chain amino acids (BCAAs) are particularly required for cell growth, *S. aureus* being auxotrophic for leucine and valine. Depletion of BCAAs results in the downregulation of CodY, a global transcriptional regulator involved in a range of cellular processes, including biofilm formation (Kaiser *et al.*, 2015). Amino acid permeases, such as the putative amino acid permease gene studied here, are membrane permeases that transport amino acids into the cell. It is clear that amino acid metabolism plays some role in the establishment of biofilms, however further work into the number of amino acid permeases that remain uncharacterised would certainly be warranted (Zhu *et al.*, 2007).

Finally, the hypothetical protein studied in this section, while uncharacterized, encoded the hypothetical protein YP_494229.1, matching a protein family HMM PF01957 (Coordinators, 2016) , an NfeD-like protein, a gene frequently associated with genes encoding stomatin-like proteins (or slipins) (Green, Lower and Young, 2009). This is a widely expressed mitochondrial inner membrane protein, putatively thought to regulate mitochondrial biogenesis and function (Christie *et al.*, 2011). Another interesting avenue for future work would be to further elucidate the role of this and other genes expressing stomatin-like proteins.

However, when these complemented strains were tested for biofilm formation alongside their transposon mutant counterparts, no significant difference was observed (Figure 19). This may be due to the high degree of error in the crystal violet assay, given the assays tendency for the biofilm to be disrupted by the washing steps, an explanation that aligns with the fact that transposon mutants and the JE2 wild type are comparable biofilm producers in this dataset (Figure 19). Given more time, repeating the complementation assay would, in part, allow more solid conclusions to be drawn from these data.

CONCLUSIONS

In the first of the three projects that make up this body of work, an attempt was made at applying metagenomic principles to the unique situation of a ward relocation. However, the microbial diversity recovered from glycerol stocks displayed markedly different population dynamics to what would be expected in clinical environmental swabs. Selective agar screening revealed the extent to which the selective media used was inappropriate for a metagenomic study, with *Pseudomonas spp.* having been recovered substantially less frequency than expected, with Enterococci almost ubiquitously producing lawns from glycerol stocks from which very little/no *Pseudomonas spp.* or *Staphylococcus spp.* were isolated from. This serves as a cautionary tale of the importance of comprehensive experimental design, particularly prior to time-sensitive projects.

To investigate the efficacy of approaching nosocomial outbreaks from a phenotype-first prospective (with a view toward elucidating potential new candidate genes capable of playing a role in epidemics), a cohort of 207 MRSA-ST228 isolates (also known as the South German Epidemic strain or the Italian clone); a multiple-antibiotic resistant, gentamicin resistant SCC*mec*-I strain of MRSA from CC5 (Mendes *et al.*, 2012), responsible for a temporally isolated outbreak in a Swiss hospital in 2008. Subsets of this cohort were screened for changes in several important phenotypes in HA-MRSA colonisation, dispersal and virulence, including biofilm formation, PSM and α-toxin production and bacteriophage susceptibility. It is hoped that any changes of these phenotypes of samples that were isolated during outbreak periods can be used to highlight potential candidate phenotypes that contributed to the sudden outbreak.

The second collection studied was a cohort of MRSA -ST239, a globally dispersed and highly persistent ST; a hybrid of descendants of two major lineages, CC8 and CC30, deemed responsible for numerous epidemics in healthcare environments worldwide (Smyth *et al.*, 2010), accounting for over 90% of HA-MRSA in China, Turkey, Thailand and much mainland Asia (Lahiri *et al.*, 2015). Regarding the global ST239, the collection used was of 95 Turkish isolates taken since, given the countries placement between Europe and Asia, this collection provided previously published insights into the spread of this clone outside Europe (Smyth *et al.*, 2010). Given promising inferences gained from this already fully sequenced collection in

terms of toxin and adhesion production (M. Laabei *et al.*, 2014b), biofilm and protease production (phenotypes that have yet to be studied in this collection) were analysed and potentially significant associations between phenotype and certain candidate SNPs were established. After screening both biofilm production and protease activity of the ST239 cohort, a top down GWAS approach was then be carried out in an attempt to elucidate the genes involved. However, the data gathered for protease activity suffered either from intrinsic limitations to the sensitivity of the method used, or due to inherent wild type differences in protease production between ST239 and the USA300_FPR3757 backgrounds. The study of ST239 biofilm formation identified five candidate genes associated with biofilm formation (*mecA*, *sgaT, ccrB, aap* and *hyp*). However, in the complementation analysis that followed, the transposon mutants used were unaccountably no different in their ability to produce biofilms than the JE2 wild type, suggesting that this experiment requires repeating for meaningful conclusions to be gleaned.

Medical microbiology has made substantial gains as a direct consequence of the introduction of next-generation sequencing technologies in the 2000s, with whole genome sequencing playing critical roles in epidemiology, detecting virulence factors and studying antibiotic resistance within pathogenic species (Padmanabhan *et al.*, 2013). The post-genomic era holds great promise, with further advances in genomic technologies heralded as a vital step towards solving some of our most urgent medical challenges.

FUTURE WORK

Aim 1: Survey changes to the microbial burden of a burns unit caused by its re-location

The task of monitoring the environmental microbial load of the Bristol paediatric burns unit was a time-sensitive project and, unfortunately, the samples collected did not reflect the true diversity of the microbiome of either the ward environment or clinical staff. However, should another opportunity arise in which a long-established clinical environment moves geographically, the shotgun metagenomic screening of fomites, fixed surfaces and clinical staff pre- and post-relocation is still of great benefit. As our results demonstrate, careful consideration is needed when determining how samples intended for use in metagenomic surveys are collected and prepared. While sampling and sample preparation vary widely in metagenomic studies, a number of common factors are important when considering experimental design. For instance, if large amounts of host DNA can be expected in sample,

fractionation or selective lysis may be necessary to ensure that the microbial sequences are not overwhelmed (Thomas, Gilbert and Meyer, 2012a). Additionally, in instances where swabs be used in sample collection, as it was in this project, low yield of DNA may also be a challenge. Since library production for most sequencers require micrograms of DNA, amplification of DNA material extracted from the samples may require amplification through, for instance multiple displacement amplification (MDA) (Thomas, Gilbert and Meyer, 2012a). From there, the subsequent DNA sequencing, annotation, statistical analysis and metadata analysis of clinical samples can be carried out.

While, in the case of the Bristol burns ward, the scope of the investigation was limited to coagulase negative and coagulase positive staphylococci, a broader metagenomic study of any clinical environment that encompasses a wider range of organisms (for instance, the ESKAPE pathogens) might also yield some insights into the transmission of nosocomial pathogens that can then be used to inform, and potentially reform, current infection control practices.

Aim 2: Survey changes to the microbial burden of a burns unit caused by its re-location

While the project studying ST228 isolates from Lausanne Hospital did not yield any significant results, there were a number of experimental flaws that may have led to this. For instance, further study of the variation of biofilm formation for the entire strain collection with sufficient biological and experimental repeats is warranted, especially if a crystal violet staining assay is used to quantify biofilm development, given the variability of the assay. Indeed, testing the entire collection for all the phenotypes studied would be of great benefit. Additionally, only two very closely related phage were used to test bacteriophage resistance. Given the abundance and variety of bacteriophage, a survey of this scale cannot be used to determine whether resistance to bacteriophage was associated with the outbreak. Further study of this collections resilience against a variety of distantly related bacteriophage would be necessary to draw any meaningful conclusions.

It might also be interesting to explore the action of any small RNA molecules or RNA-RNA interactions at a post-transcriptional level that might be associated with the outbreak. RNA mediated changes in for these strains could be quantified via reverse transcription PCR (RT-PCR) in different *in vitro* conditions. Additionally, protease production, through an assay

similar to the one presented in Chapter 3, alongside surveying other phenotypes, such as fibrinogen-binding and fibronectin-binding (Stemberk *et al.*, 2014)

Aim 3: Determining novel candidate genes associated with protease production and biofilm formation through a GWAS of a population of ST239

A high-throughput solution to the quantification of exogenous proteases from bacterial supernatant was developed during this project. While the milk agar assay used here was helpful in determining qualitatively which isolates showed unusually high biofilms, measurements were prone to human error. A protease assay suitable for bacterial supernatant that is both high-throughput and accurate would be ideal in scenarios such as this. An example of how this might be achieved could be through measuring amino acids liberated by protease activity with chemical reagents that undergo a colormetric change in the presence of particular amino acids, such as Folin reagent, as was attempted here. While in this instance the source of the unreliability of the data produced could not be determined, adequate troubleshooting in future attempts might lead to a more efficient way of studying protease production.

Five candidate genes were found to be potentially associated with biofilm production in the ST239 dataset, *mecA*, *ccrB*, *sgaT*, *aap* and *hyp.* While the cloning required for complementation was thought to be successful, this step may need to be repeated before the relevance of these loci is discounted. If any genes, after complementation, still seem likely to have a true association with biofilm formation, protein purification and characterisation of the proteins encoded by these genes in the best and worst biofilm forming isolates of this cohort would be of benefit. This may include simple assays such as western blotting, or through techniques such as Nuclear Magnetic Resonance (NMR) spectroscopy.

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APPENDIX

Appendix 1 – Primers designed from complementation of biofilm associated candidate genes

Gene	Strand	Sequence (5' to 3')	Restriction site	Amplicon length (bp)
ccrB	Forward	GGCTGCGAATTCCGAGGAGATTGTAAGATG	EcoRI	817
ccrB	Reverse	GTCGGCGGTACCAATACGATGGACTCGGTG	KpnI	
sgaT	Forward	CGTTTCGAATTCAAGGAGGTTCGACCTATG	EcoRI	1575
sgaT	Reverse	CCCGAGGGTACCGGCATTCACTCAATATGTGAC	KpnI	
aap	Forward	TGCCCAGAATTCAACGAAGGAGTATTTATG	EcoRI	2099
aap	Reverse	GCCGAGGGTACCCATTGATTTATGTCCCAGCC	KpnI	
hyp	Forward	GGCTTTGAATTCCTAAACATGGTATCGCTCC	EcoRI	948
hyp	Reverse	GCAGACGGTACCTACCTCTGTTGACTCCCTC	KpnI	
mecA	Forward	CTCAAGGAATTCAGGAGGATATTGATG	EcoRI	2155
mecA	Reverse	GATCCGGGTACCGTGAAGCAACCATCGTTACGG	KpnI	

Appendix 2 - Environmental strains collected from Frenchay Hospital Barbara Russell Children's burns unit (Before move)

Room collected from	Items swabbed	Growth after initial plating on TSA	Sample reference
Burns clinic (room 3)	Cupboard handles	Many small colonies	BRCU1-CH1
Burns assessment room	Cupboard handles	Few small colonies	BRCU1-CH2
Dressing changing room	Cupboard handles	Many large colonies	BRCU1-CH3
Burns assessment room	Touch screen monitors	Many small colonies	BRCU1-TSM1
Burns reception	Touch screen monitors	Lawn	BRCU1-TSM2
HDU reception	Touch screen monitors	Lawn	BRCU1-TSM4
Burns clinic (room 3)	Computer monitors	Mixed lawn	BRCU1-CM1
Burns assessment room	Computer monitors	Many small colonies	BRCU1-CM2
Burns reception	Computer monitors	Few large colonies	BRCU1-CM4
Burns clinic (room 3)	Keyboards and mouse	Lawn	BRCU1-K1
Burns assessment room	Keyboards and mouse	Mixed lawn	BRCU1-K2
Mobile laptop	Keyboards and mouse	Lawn	BRCU1-K3
Burns reception	Keyboards and mouse	Mixed lawn	BRCU1-K4
Nurse station 1	Keyboards and mouse	Many small colonies	BRCU1-K5
Nurse station 2	Keyboards and mouse	Mixed lawn	BRCU1-K6
Starfish ward (TV)	Remote controls	Lawn	BRCU1-R1
Burns assessment room	Remote controls	Lawn	BRCU1-R2
Playroom (TV)	Remote controls	Few large colonies	BRCU1-R4
Burns clinic (room 3)	Tap/Shower head	Lawn	BRCU1-TH1

Burns bathroom sink	Tap/Shower head	Lawn	BRCU1-TH3
Burns bathroom shower	Tap/Shower head	Lawn	BRCU1-TH4
Burns bathroom sink	Sinks	Mixed lawn	BRCU1-S3
Burns bathroom bath	Sinks	Few large colonies	BRCU1-S4
Burns room 2	Sinks	Lawn	BRCU1-S5
Burns room 2	Bed linen	Many small colonies	BRCU1-BL2
Burns room 3	Bed linen	Lawn	BRCU1-BL4
Physiotherapy room	Bed linen	Lawn	BRCU1-BL5
Burns clinic (room 3)	Bed posts	Few small colonies	BRCU1-BP1
Starfish ward	Bed posts	Mixed lawn	BRCU1-BP4
Burns clinic (room 1)	Bed posts	Lawn	BRCU1-BP6
Physiotherapy room	Bike handles	Mixed lawn	BRCU1-PT1
Physiotherapy room	Large exercise ball	Many large colonies	BRCU1-PT4
Physiotherapy room	Balance beam	Lawn	BRCU1-PT5
Playroom	Book (Grunter)	Mixed lawn	BRCU1-Book3
Corridor	Book (Ex Files)	Mixed lawn	BRCU1-Book4
Corridor	Magazine (Marie Claire)	Mixed lawn	BRCU1-Book5
Burns clinic (room 3)	Toy (Car)	Mixed lawn	BRCU1-Toy1
Burns assessment room	Toy (Small ball)	Lawn	BRCU1-Toy2
Burns bathroom	Toy (Boat)	Many large colonies	BRCU1-Toy3
Playroom	Toy (Kitchen)	Lawn	BRCU1-Toy4
Playroom	Toy (Castle)	Lawn	BRCU1-Toy5
School	Touch screen tablets (Black)	Lawn	BRCU1-TST1
Playroom	Touch screen tablets (Anti-microbial case)	Lawn	BRCU1-TST2
Playroom	Touch screen tablets (No case)	Many large colonies	BRCU1-TST3
School	Touch screen tablets (Red)	Lawn	BRCU1-TST4
Nurses station 2	Office desk	Mixed lawn	BRCU1-D3
Main desk	Office desk	Mixed lawn	BRCU1-D4
Burns clinic main desk	Office desk	Mixed lawn	BRCU1-D5
Burns assessment room	Worktop	Lawn	BRCU1-W1
Dressing changing room	Worktop	Many small colonies	BRCU1-W2
HDU reception	Worktop	Mixed lawn	BRCU1-W3
School	Worktop	Mixed lawn	BRCU1-W4
Burns clinic staff room	Worktop	Mixed lawn	BRCU1-W5
Burns clinic (room 1)	Worktop	Many small colonies	BRCU1-W6
HDU reception	Play surfaces	Many small colonies	BRCU1-P1
Playroom	Play surfaces	Mixed lawn	BRCU1-P2
Corridor	Play surfaces	Lawn	BRCU1-P3
Sensory room	Play surfaces	Mixed lawn	BRCU1-P4
Waiting area	Play surfaces	Mixed lawn	BRCU1-P5

School	Play surfaces	Lawn	BRCU1-P6
Burns clinic (room 3)	Door handles	Many small colonies	BRCU1-DH1
Burns bathroom	Door handles	Many small colonies	BRCU1-DH3
Burns room (2)	Door handles	Many large colonies	BRCU1-DH4
Burns clinic (room 3)	Phones	Mixed lawn	BRCU1-Phn1
Burns assessment room	Phones	Mixed lawn	BRCU1-Phn2
Burns main desk	Phones	Mixed lawn	BRCU1-Phn3
Nurses station 1	Phones	Lawn	BRCU1-Phn4
Burns clinic staff room	Phones	Lawn	BRCU1-Phn5
Burns clinic main desk	Phones	Mixed lawn	BRCU1-Phn6

Appendix 3 - Strains collected from Frenchay Hospital Barbara Russell Children's burns unit staff (Before move)

Type of worker	**Hand/nose/ID card**	**Postcode**	**Growth after initial plating on TSA**	**Sample reference**
Nurse	Hand	BS1	Lawn	N1H
Nurse	Nose	BS1	Many colonies	N1N
Nurse	ID card	BS1	Many colonies	N1C
Nurse	Hand	BS16	Lawn	N2H
Nurse	Nose	BS16	Many colonies	N2N
Nurse	ID card	BS16	Many colonies	N2C
Nurse	Hand	Not given	Lawn	N4H
Nurse	Nose	Not given	Lawn	N4N
Nurse	ID card	Not given	Lawn	N4C
Nurse	Hand	Not given	Lawn	N5H
Nurse	Nose	Not given	Lawn	N5N
Nurse	ID card	Not given	Lawn	N5C
Nurse	Hand	BS15	Many colonies	N6H
Nurse	Nose	BS15	Lawn	N6N
Nurse	ID card	BS15	Lawn	N6C
Nurse	Hand	BS35	Lawn	N7H
Nurse	Nose	BS35	Few colonies	N7N
Nurse	ID card	BS35	Lawn	N7C
Nurse	Hand	BS36	Lawn	N8H
Nurse	Nose	BS36	Lawn	N8N
Nurse	ID card	BS36	Lawn	N8C
Nurse	Hand	BS36	Many colonies	N9H
Nurse	Nose	BS36	Lawn	N9N
Nurse	ID card	BS36	Lawn	N9C
Nurse	Hand	BS10	Lawn	N10H
Nurse	Nose	BS10	Lawn	N10N
Nurse	ID card	BS10	Few colonies	N10C

Doctor	Nose	BS9	Lawn	D2N
Doctor	Hand	BS39	Lawn	D3H
Doctor	Nose	BS39	Lawn	D3N
Doctor	Hand	BS36	Lawn	D7H
Doctor	Nose	BS36	Lawn	D7N
Doctor	ID card	BS36	Lawn	D7C
Doctor	Hand	BS6	Lawn	D8H
Doctor	Nose	BS6	Lawn	D8N
Doctor	ID card	BS6	Lawn	D8C
Doctor	Hand	Not given	Lawn	D9H
Doctor	Nose	Not given	Many colonies	D9N
Doctor	ID card	Not given	Many colonies	D9C
Miscellaneous staff	Hand	GL12	Lawn	H1H
Miscellaneous staff	Nose	GL12	Few colonies	H1N
Miscellaneous staff	ID card	GL12	Few colonies	H1C
Miscellaneous staff	Hand	BS16	Lawn	H3H
Miscellaneous staff	Nose	BS16	Lawn	H3N
Miscellaneous staff	ID card	BS16	Lawn	H3C
Miscellaneous staff	Hand	BS16	Lawn	H7H
Miscellaneous staff	Nose	BS16	Lawn	H7N
Miscellaneous staff	ID card	BS16	2 colonies only	H7C
Miscellaneous staff	Hand	BS8	Lawn	H8H
Miscellaneous staff	Nose	BS8	Lawn	H8N
Miscellaneous staff	ID card	BS8	Lawn	H8C

Appendix 4 - Strains collected from the new location for the Bristol Children's burns unit, Bristol Royal Hospital for Children (Before the move)

Items swabbed	**Room**	**Sample reference**
Sink handle	Treatment room	TR1
Inside tap nossle	Treatment room	TR2
Sink basin	Treatment room	TR3
Worktop	Treatment room	TR4

Green cupboard	Treatment room	TR5
Door handles	Treatment room	TR6
Sink handle	Large room	LR1
Inside tap nossle	Large room	LR2
Sink basin	Large room	LR3
Shower tap	Large room	LR4
Window ledge	Large room	LR5
Power switches	Large room	LR6
Sink handle	Media room 1	MR1
Sink basin	Media room 1	MR2
Power swiitches	Media room 1	MR3
Cupboard handles	Media room 1	MR4
Bath	Bathroom	BTL1
Bath buttons/dials	Bathroom	BTL2
Shower	Bathroom	BTL3
Nappy changing unit	Bathroom	BTL4
Sink handle	Bathroom	BTL5
Sink basin	Bathroom	BTL6
Desk	Reception	RP1
Desk	Reception	RP2
Phone	Reception	RP3

Appendix 5- Strains collected from a surgical suite before and after a surgical procedure, as well as after cleaning through "fogging" within the new location for the Bristol Children's burns unit, Bristol Royal Hospital for Children (After move)

Items swabbed	**Room**	**Tine of swabbing**	**Sample reference**
Theatre trolley top (metal)	Burns theatre	Before surgery	BT2
Theatre trolley top (metal)	Burns theatre	Before surgery	BT3
Patient arm 'L' boards	Burns theatre	Before surgery	BT6
In theatre computer keyboard and mouse	Burns theatre	Before surgery	BT7
In theatre computer monitor screen	Burns theatre	Before surgery	BT8
In theatre computer fan grill	Burns theatre	Before surgery	BT9
Scrubbing up bench	Burns theatre	Before surgery	BT11
Operating table bolster cushions	Burns theatre	Before surgery	BT16
In theatre dressing trolley handle	Burns theatre	Before surgery	BT19
Tourniquet cuffs	Burns theatre	Before surgery	BT20
Patient arm 'L' boards	Burns theatre	After surgery	AT3
Anaesthetic machine buttons	Burns theatre	After surgery	AT5
Patient arm 'L' boards	Burns theatre	After surgery	AT6
In theatre computer keyboard and mouse	Burns theatre	After surgery	AT7

In theatre computer fan grill	Burns theatre	After surgery	AT9
Scrubbing up bench	Burns theatre	After surgery	AT11
Scrub sink tap handles	Burns theatre	After surgery	AT13
In theatre dressing trolley top	Burns theatre	After surgery	AT18
In theatre dressing trolley handle	Burns theatre	After surgery	AT19
Tourniquet cuffs	Burns theatre	After surgery	AT20
Diathermy machine buttons	Burns theatre	After fogging	F10
Scrubbing up bench	Burns theatre	After fogging	F11
In theatre trolley top	Burns theatre	After fogging	F18
In theatre trolley handles	Burns theatre	After fogging	F19
Bench top	Anaesthetic room	Before surgery	BA1
Room telephone	Anaesthetic room	Before surgery	BA2
Cupboard handles	Anaesthetic room	Before surgery	BA3
Sink tap handle	Anaesthetic room	Before surgery	BA4
Sink plug hole	Anaesthetic room	Before surgery	BA5
Sink tap nozzle	Anaesthetic room	Before surgery	BA6
Anaesthetic machine buttons	Anaesthetic room	Before surgery	BA7
Sink plug hole	Recovery room	Before surgery	BR2
Sink tap	Recovery room	Before surgery	BR3
Computer keyboard/mouse	Recovery room	Before surgery	BR4
Computer screen	Recovery room	Before surgery	BR5
Computer fan grill	Recovery room	Before surgery	BR6